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OF

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XXV. Experimental Typhoid Carriers. VI. The Reaction and Physiology of the Hepatic Duct and Cystic Bile of Various Laboratory Animals (Journal of Infectious Diseases, May–June, 1921, vol. XXVIII, pp. 510–541), by N. M. Neilson and K. F. Meyer.

XXVI. Experimental Typhoid Carriers. VII. The Bacteriostatic and Germicidal Properties of Bile (Journal of Infectious Diseases, May–June, 1921, vol. XXVIII, pp. 542–587), by N. M. Neilson and K. F. Meyer.

XXVII. Experimental Typhoid Carriers. VIII. The Influence of the H-ion Concentration on the Growth of B. typhosus in Mediums Containing Bile or Bile Salts (Journal of Infectious Diseases, May–June, 1921, vol. XXVIII, pp. 588–605) by P. Schoenholz and K. F. Meyer.

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Iron and Arsenic as Influencing Blood Regeneration Following Simple Anemia

~~VI.~~ Negative Influence of Familiar Drugs on the Curve of Hemoglobin Regeneration Following Hemorrhage

G. H. WHIPPLE AND F. S. ROBSCHEIT
SAN FRANCISCO

IRON AND ARSENIC AS INFLUENCING BLOOD REGENERATION FOLLOWING SIMPLE ANEMIA

VI. NEGATIVE INFLUENCE OF FAMILIAR DRUGS ON THE CURVE OF HEMOGLOBIN REGENERATION FOLLOWING HEMORRHAGE *

G. H. WHIPPLE and F. S. ROBSCHEIT
SAN FRANCISCO

Is iron of value in the treatment of anemia? Is iron absorbed from the intestine when given in organic or inorganic form? Such questions lead to fields of discussion over which has risen the smoke of polemics and arguments without end. We believe that some of these difficulties may be cleared away by careful definition of terms. Our experiments deal with secondary anemia due to hemorrhage and not with chlorosis or any other form of anemia. We have controlled certain diet factors which can influence profoundly the hemoglobin regeneration in this form of secondary anemia. We suggest that our critics control these diet factors before any great emphasis is placed upon drug factors. It is now established that diet factors are concerned in the rapid regeneration of hemoglobin, therefore, these facts must be considered in evaluating any reaction in conditions of anemia.

Chlorosis has been mentioned so frequently in this discussion and others that we feel it necessary to state that in our opinion this condition does not belong in this group of simple secondary anemias. Whether this disease reacts favorably to iron therapy is not germane to this discussion. It seems to be a disease which is becoming much less frequent in many communities due to modified dietary or living conditions or other unknown factors. It would be of interest to collect accurate data on the influence of diet factors alone in this disease.

We do not think it is necessary to review the literature of anemia and iron therapy. It may be admitted that we have not tested all the various forms of iron which are being used in the treatment of anemia, but it seems to us that this is not necessary. We are able to show that many of the iron preparations commonly prescribed are inert, or, at least, much less potent than simple diet factors. The burden of proof now rests with those who claim that one or more iron preparations are potent in anemia under controlled diet conditions.

Some experimental work has been done in secondary anemia to show whether or not iron compounds are potent in stimulating the regenera-

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* From the George Williams Hooper Foundation for Medical Research, University of California Medical School.

tion of hemoglobin. In much of this work the anemia was produced by drugs which destroy the red cells in the body and leave the freed hemoglobin to be stored or used in any way by the endogenous body mechanism. This type of anemia must not be confused with the type used in our experiments—that is, anemia due to simple hemorrhage. This destruction of red cells in the body may be associated with a storage of hemoglobin radicles somewhere in the body which may be capable of influencing subsequent hemoglobin regeneration. We hope to report on this type of hemoglobin regeneration in the near future.

Arsenicals were used in some of our experiments. It seemed best to include some of this group in this paper because their use in certain forms of anemia is well recognized. Both Fowler's solution and sodium cacodylate are inert under the conditions of these experiments.

Objection may be raised to the use of dogs in these experiments because they are carnivorous animals. The dogs used by us were raised in our kennels and have been omnivorous since weaning. The diet on which they thrive is a mixture of food scraps obtained from the University Hospital. It contains cooked bones, meat, bread, potatoes, rice, etc. This mixed diet obtains in all resting periods between experiments. Unless otherwise noted, these animals are active, healthy and normal in all respects.

EXPERIMENTAL OBSERVATIONS

The general method used in all these experiments has been fully discribed in the first paper of this series.[1] Unless otherwise stated, the plasma volume has been directly determined by the nontoxic dye, "brilliant vital red." [2] This dye has been furnished us through the courtesy of Dr. H. M. Evans of the department of anatomy, and we are indebted to him for much advice and council. The blood is received into isotonic sodium oxalate solution, and the ratio of cells and plasma is determined in calibrated centrifuge tubes. The hemoglobin is determined by a modification of the Palmer method described recently by one of us (F.S.R.).[3] Red and white counts are made in the usual manner from blood obtained by venous puncture. For other details of this work, we refer the reader to the first paper of this series.[1]

Paper V of this series [4] records many experiments in which Blaud's pills were added to various diets. We wish to refer to this paper for the details of these experiments. Blaud's pills were inert under these experimental conditions. It seemed necessary to test out other drugs

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1. Whipple, Hooper and Robscheit: Am. J. Physiol. **53**:151, 1920.
2. Hooper, Smith, Belt and Whipple: Am. J. Physiol. **51**:205, 1920.
3. Robscheit: J. Biol. Chem. **41**:209, 1920.
4. Hooper, Robscheit and Whipple: Am. J. Physiol. **53**:263, 1920.

under these same conditions, and the charts giving these data are given below.

TABLE 82.—BLOOD REGENERATION—FERRIC CITRATE

Dog 18-114. Bull mongrel. Female. Adult

Date, 1920	Pigment Volume = Hb. per Cent. Times Blood Volume	Blood Volume	Plasma Volume	R. B. C. Volume	R. B. C. Hematocrit	Hb.	Color Index	R. B. C., Million	W. B. C.	Weight	Blood per Kilogram	Remarks
		C.c.	C.c.	C.c.	%	%				Kg.	C.c.	
7/ 8	2,078	1,625	754	854	52.4	128	0.72	8,8	12,4	12.25	132	
7/ 8	Diet: Bread and milk											
7/ 9 7/10	Bled 406 c.c. Bled 406 c.c.											
7/12	806	1,262	902	348	27.6	64	0.82	3,9	8,2	11.95	105	
7/12	Diet: 150 gm. bread, 500 c.c. milk, 1 c.c. of 5% solution of ferric citrate subcutaneously daily											
7/20	1,188	1,383	858	491	35.5	86	0.94	4,6	12,2	11.95	115	
7/28	1,215	1,380	836	520	37.6	88	0.80	5,5	9,8	11.70	118	* Slight
8/ 3	1,225	1,340	822	523	39.0	91	0.79	5,8	9,0	11.50	116	* Slight
8/13	1,130	1,410	846	544	38.5	80	0.73	5,6	8,2	11.65	121	* Slight
8/20	1,344	1,325	768	574	43.3	101	0.85	6,0	7,4	11.35	117	* Slight
8/27	1,493	1,440	772	646	44.9	104	0.84	6,2	11,0	11.20	128	* Slight
9/ 3	1,428	1,498	850	629	42.0	95	0.75	6,3	10,4	11.05	135	* Poik.
9/10	1,405	1,388	774	599	43.2	101	0.80	6,3	8,4	10.65	130	* Poik.
9/10	Diet: Mixed, with extra food. (No ferric citrate given)											
9/17	1,693	1,693	985	691	40.8	100	0.73	6,9	6,4	13.55	125	* Poik.+†
9/24	1,653	1,798	1,053	722	40.1	92	0.73	6,3	6,8	15.00	120	* Poik.+†

* Poikilocytosis of red blood cells.
† Food poor in meat.

Table 82 shows the curve of blood regeneration of a standard dog on a bread and milk diet given ferric citrate subcutaneously. It may be stated that the same reaction would be expected in the same dog under identical conditions without the ferric citrate. We can refer to other experiments on this same dog which show the expected favorable and rapid blood regeneration on diets of meat and liver. Table 49 of this series [5] shows the curve of blood regeneration on a diet of cooked meat scraps. The return to normal was almost complete in three weeks. Also Table 61 of this series [6] shows the curve of blood regeneration on a diet of cooked beef liver. The return to normal was complete in two weeks and somewhat above the initial level in three weeks.

This favorable blood regeneration on this diet of meat or liver is in contrast to the slow reaction expected on bread and milk or actually recorded in Table 82 with bread and milk plus ferric citrate.

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5. Whipple, Robscheit and Hooper: Am. J. Physiol. **53**:238, 1920.
6. Whipple, Robscheit and Hooper: Am. J. Physiol. **53**:252, 1920.

Several interesting points in this experiment should be noted. The blood per kilo is a very high figure, on the average. This is usually found in very active dogs which are somewhat below normal in weight. This dog lost some weight during the bread and milk feeding and the plasma volume likewise decreased as is common under such conditions. This accounts, in part, for some of the increase in red hematocrit and hemoglobin, but is more accurately evaluated in the pigment volume figures. When the dog is put on forced feeding, there is a remarkable gain in weight and plasma volume and an actual fall in red hematocrit and hemogoblin. The pigment volume, however, shows the true values for hemoglobin production. A hasty survey of these figures might lead one to erroneous conclusions unless the facts as given are carefully weighed. This type of experiment shows, too, the necessity of blood plasma measurements.

TABLE 83.—BLOOD REGENERATION—FERRIC CITRATE

Dog 19-95. Bull mongrel. Male. Adult

Date, 1920	Pigment Volume = Hb. per Cent. Times Blood Volume	Blood Volume	Plasma Volume	R. B. C. Volume	R. B. C. Hematocrit	Hb.	Color Index	R. B. C., Million	W. B. C.	Weight	Blood per Kilogram	Remarks
		C.c.	C.c.	C.c.	%	%				Kg.	C.c.	
7/8	1,570	1,412	754	644	45.6	111	0.79	7,0	7,4	12.25	115	* Slight
7/8	Diet: Bread and milk											
7/9 7/10	Bled 353 c.c. Bled 353 c.c. No distress											
7/12	722	1,052	741	301	28.6	68	0.86	4,0	11,0	12.05	87	* Slight
7/12	Diet: 150 gm. bread, 500 c.c. milk, 1 c.c. of 5% solution ferric citrate subcutaneously daily											
7/20	1,133	1,295	814	469	36.2	87	0.87	5,0	12,2	11.80	110	* Slight
7/28	1,190	1,264	749	508	40.2	94	0.78	6,0	10,8	11.80	107	* Slight
8/3	1,256	1,256	702	542	43.2	100	0.84	6,0	7,2	11.75	107	* Slight
8/13	1,141	1,197	672	513	42.9	95	0.79	6,0	17,0	11.75	102	* Slight
8/20	1,215	1,225	711	502	41.0	99	0.85	5,8	12,0	11.55	106	* Slight
8/27	1,185	1,212	695	505	41.7	98	0.71	6,9	5,8	11.65	104	* Poik. –
9/3	1,273	1,303	730	561	43.0	98	0.72	6,8	6,4	11.50	113	* Poik. ±
9/10	1,165	1,202	709	481	40.0	97	0.81	6,0	7,2	11.35	106	* Poik +
9/10	Diet: Mixed, with extra meat. (No ferric citrate given)											
9/17	1,632	1,497	808	674	45.0	109	0.83	6,6	6,6	13.75	109	* Slight

* Poikilocytosis of red blood cells.

Table 83 shows the influence of ferric citrate given subcutaneously. We are able to refer to other experiments on this same animal which have been published. Table 3 of this anemia series[7] gives the curve of blood regeneration on a mixed diet. It is clear in this experiment that blood regeneration was slow until meat was added in liberal amounts. The curve of regeneration finally returned to normal. Table 41 gives the blood regeneration curve on a liberal diet of rice, potatoes and milk. It is clear that these curves, if anything, are better than the curve of Table 83. We have established the fact that this rice-potato-milk diet is slightly more favorable for blood regeneration than a simple bread and milk diet.

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[7] Whipple, Hooper and Robscheit: Am. J. Physiol. **53:**159, 1920.

These control experiments under identical conditions make it clear that the ferric citrate under these experimental conditions is inert. The curve of regeneration has not been influenced by the administration of this drug.

TABLE 84.—Blood Regeneration—Ovoferrin

Dog 20-3. Bull mongrel. Male, Adult

Date, 1920	Pigment Volume = Hb. per Cent. Times Blood Volume	Blood Volume	Plasma Volume	R. B. C. Volume	R. B. C. Hematocrit	Hb.	Color Index	R. B. C., Million	W. B. C.	Weight	Blood per Kilogram	Remarks
		C.c.	C.c.	C.c.	%	%				Kg.	C.c.	
7/29	1,588	1,495	766	715	47.8	106	0.77	6,9	7,6	13.60	110	* Slight
7/29	Diet: Bread and milk											
7/30 7/31	Bled 374 c.c. Bled 374 c.c.											
8/ 2	634	1,177	884	281	23.9	54	0.69	3,9	8,2	13.05	90	* Slight
8/ 3	Diet: 150 gm. bread, 500 c.c. milk, 1 teaspoonful ovoferrin daily											
8/13	1,065	1,287	784	490	38.1	83	0.81	5,1	8,6	12.35	104	* Slight
8/13	Diet: 150 gm. bread, 500 c.c. milk, 2 teaspoonfuls ovoferrin daily											
8/20	1,148	1,247	750	490	39.3	92	0.79	5,8	7,8	12.20	102	* Slight
8/27	1,057	1,185	706	468	39.5	89	0.75	5,9	6,6	11.65	102	* Slight
9/ 3	1,075	1,273	783	477	37.5	84	0.96	4,9	6,4	11.55	110	* Slight
9/ 3	Diet: Mixed, with extra food (meat ++). No ovoferrin											
9/10	1,180	1,382	896	475	34.4	85	0.85	5,0	6,6	13.00	106	* Slight
9/17	1,410	1,658	1,053	588	35.5	85	0.83	5,1	6,2	15.00	110	* Slight
9/24	1,915	1,982	1,120	842	42.5	97	0.83	5,8	6,4	16.80	118	

* Poikilocytosis of red blood cells.

Table 84 may, at first sight, be favorable to ovoferrin as influencing the curve of blood regeneration. On careful analysis, however, we note very little, if any, change which may be attributed to the drug. Compare Table 84 with Table 92, an experiment of similar type during which sodium cacodylate was administered. There is little difference in reaction. Another experiment on this same dog is recorded (Table 86) in which a salt mixture containing a small amount of iron was given. This salt mixture seems to exert a somewhat favorable influence—a much more decided influence than the iron alone. Therefore, this minimizes the actual influence of the iron on the hemoglobin regeneration. The ovoferrin (Ferri vitellinum syntheticum) was obtained from the University Hospital.

Table 84 shows a sharp jump in hemoglobin, red hematocrit and pigment volume during the first ten days following the double bleeding. This is often observed after two bleedings, and has less significance than when following three bleedings, which tend to lessen this "reserve factor." We believe this reaction has little relation to the drug. During the next three weeks on the drug the pigment volume remains unchanged, although there are slight fluctuations in red hematocrit and hemoglobin. This means that the "maintenance factor" alone is being supplied. A change to a heavy mixed diet with cooked meat brings about little change in hemoglobin and hematocrit values, but the great gain in weight is associated with much increase in blood volume and actually the pigment volume gives the true picture which is a rapid increase in actual amount of formed hemoglobin in the body. The pigment volume almost doubles in this short period, associated with a gain in weight of 5 kilos. This shows again the necessity of blood volume measurement in work of this sort and shows the comparative value of iron and meat as influencing hemoglobin formation in the body under these conditions. We can attribute little value to the drug but the meat diet at once shows a striking reaction.

TABLE 85.—BLOOD REGENERATION—OVOFERRIN

Dog 20-2. Bull mongrel. Male. Young adult

Date, 1920	Pigment Volume = Hb. per Cent. Times Blood Volume	Blood Volume	Plasma Volume	R. B. C. Volume	R. B. C. Hematocrit	Hb.	Color Index	R. B. C., Million	W. B. C.	Weight	Blood per Kilogram	Remarks
		C.c.	C.c.	C.c.	%	%				Kg.	C.c.	
7/29	2,001	1,715	796	904	52.6	117	0.79	7,4	7,2	15.15	113	* Slight
7/29	Diet: Bread and milk											
7/30 7/31	Bled 430 c.c. Bled 430 c.c.											
8/2	898	1,261	863	392	31.1	71	1.10	3,2	6,8	14.45	87	
8/3	Diet: 150 gm. bread, 500 c.c. milk, 1 teaspoonful ovoferrin daily											
8/13	1,310	1,447	803	638	44.1	90	0.74	6,1	6,0	14.05	103	
8/13	Diet: 150 gm. bread, 500 c.c. milk, 2 teaspoonfuls ovoferrin daily											
8/20	1,513	1,428	786	636	44.5	106	0.88	6,0	6,8	13.76	104	* Slight
8/27	1,430	1,430	780	636	44.5	100	0.77	6,5	5,2	13.65	105	* Slight
9/3	1,670	1,535	774	742	48.3	109	0.72	7,6	6,0	13.55	113	* Slight
9/10	1,523	1,419	744	661	46.6	107	0.85	7,3	6,4	13.20	107	* Slight
9/17	1,605	1,445	765	668	46.3	111	0.83	6,7	5,8	13.20	109	* Slight
9/17	Diet: Mixed.† No ovoferrin											
9/24	1,858	1,795	965	822	45.8	104	0.80	6,5	6,0	16.00	112	* slight

* Poikilocytosis of red blood cells.
† Food poor in meat.

Table 85 shows a somewhat favorable reaction following two bleedings. The anemia level in this experiment was not sufficiently reduced, and a reaction of this type would be expected on bread and milk alone. Compare this experiment with another on the same dog (Table 90) using Fowler's solution. The change in blood volume is striking when the dog is put on a liberal mixed diet and the corresponding adjustment of hemoglobin and hematocrit values is of interest.

TABLE 86.—BLOOD REGENERATION—SALT MIXTURE *

Dog 20-3, Bull mongrel. Male. Adult

Date, 1919	Pigment Volume = Hb. per Cent. Times Blood Volume	Blood Volume	Plasma Volume	R. B. C. Volume	R. B. C. Hematocrit	Hb.	Color Index	R. B. C., Million	W. B. C.	Weight	Blood per Kilogram	Remarks
		C.c.	C.c.	C.c.	%	%				Kg.	C.c.	
10/27	1,900	1,478	736	721	48.8	129	0.93	6,9	8,8	16.55	89	
10/27	Diet: Bread and milk											
10/28 10/29	Bled 370 c.c. Bled 370 c.c. No distress											
10/31	778	1,045	732	303	29.0	74	0.95	3,9	22,6	15.55	67	
10/31	Bled 261 c.c.											
11/ 3	661	1,030	740	271	26.3	64	0.97	3,3	16,8	15.35	67	
11/ 3	Diet: 175 gm. bread, 2 gm. salt mixture,* 500 c.c. milk											
11/13	1,090	1,160	697	454	39.1	94	0.89	5,3	9,2	15.50	75	
11/20	1,580	1,421	765	643	45.2	111	0.76	7,3	9,0	15.30	93	† Slight
11/26	1,548	1,421	711	697	49.0	109	0.68	8,0	8,0	14.75	96	† Slight
12/ 3	1,545	1,400	700	686	49.0	110	0.68	8,1	6,8	14.70	95	† Slight
12/10	1,666	1,436	718	706	49.1	116	0.68	8,5	6,8	14.35	100	† Slight
12/17	1,445	1,343	674	656	48.8	108	0.74	7,3	7,4	14.15	95	† Slight
12/24	1,474	1,333	566	654	49.0	110	0.65	8,4	13,6	14.00	95	† Poik.++

Beginning dietary deficiency disease. Recovery.

* Salt mixture No. 185 (McCollum and Simmonds:[9] NaCl, 8.65 gm.; $MgSO_4$ (anhydrous), 13.30 gm.; $NaH_2PO_4H_2O$, 17.35 gm.; K_2HPO_4, 47.70 gm.; $CaH_4(PO_4)_2H_2O$, 27.00 gm.; Fe citrate, 5.9 gm.; Ca lactate, 65.0 gm. Iron equivalent to 64 mg. or 0.96 gm. daily.

† Poikilocytosis of red blood cells.

Tables 86, 87 and 88 show experiments which are much alike but present minor differences. This salt mixture (McCollum and Simmonds) contains per 2 gm. dose ferric citrate amounting to 64 mg. We note, in general, a somewhat more favorable reaction than is to be expected on this same diet without the salt mixture. This reaction is much like that observed on a liberal diet of rice, potatoes and milk. The regeneration during the first two weeks is quite rapid, but the return to normal is never complete, and the dogs show little change during subsequent weeks, the hemoglobin, red hematocrit and pigment volume showing minor fluctuations but remaining subnormal. It may well be debated that this favorable reaction is not to be attributed to the iron salts but to one or another of the many salts in this mixture. We hope to make observations on the influence of various salt mixtures with and without iron, both organic and inorganic.

Two dogs developed a characteristic dietary deficiency disease after a period of several weeks. This condition will be discussed at length in a future publication.

TABLE 87.—Blood Regeneration—Salt Mixture †

Dog 20-1. Bull mongrel. Female. Adult

Date, 1919	Pigment Volume = Hb. per Cent. Times Blood Volume	Blood Volume	Plasma Volume	R. B. C. Volume	R. B. C. Hematocrit	Hb.	Color Index	R. B. C., Million	W. B. C.	Weight	Blood per Kilogram	Remarks
		C.c.	C.c.	C.c.	%	%				Kg.	C.c.	
10/27	1,744	1,320	647	668	50.6	132	0.89	7,4	12,4	13.75	96	
10/27	Diet: Bread and milk											
10/28	Bled 330 c.c. No distress											
10/29	Bled 330 c.c. No distress											
10/31	848	1,072	732	330	30.8	79	1.01	3,9	28,6	13.35	80	
11/1	Bled 268 c.c.											
11/1	617	984	712	253	25.7	63	1.10	2,8	22,0	13.00	76	
11/3	Diet: 200 gm. bread, 2 gm. salt mixture,† 500 c.c. milk											
11/13	898	984	596	377	38.3	91	0.80	5,7	9,6	12.50	79	
11/20	1,305	1,120	570	540	48.2	117	0.68	8,6	5,2	12.10	93	
11/22	Diet: 150 gm. bread, 2 gm. salt mixture, 500 c.c. milk											
11/26	1,395	1,255	605	638	50.8	111	0.65	8,6	5,8	11.80	106	
12/3	1,315	1,190	592	592	49.75	111	0.67	8,3	8,8	11.70	102	
12/10	1,640	1,268	600	656	51.7	129	1.02	8,3	5,6	11.25	113	
12/17	1,524	1,272	579	681	53.5	120	0.73	8,2	7,0	11.00	116	* Poik.
12/17	Diet: 200 gm. bread, 2 gm. salt mixture, 500 c.c. milk											
12/24	1,445	1,214	595	607	50.0	119	0.66	9,0	5,0	11.00	110	* Poik.+
12/30	1,375	1,115	558	546	48.9	123	0.75	8,2	5,4	10.75	104	* Poik.+
1/6/20	1,141	1,120	611	499	44.5	102	0.76	6,7	5,2	11.00	102	* Poik.
1/21	940	1,075	626	439	40.8	87	0.63	6,9	5,8	10.80	100	* Poik.
1/23	Diet: 250 gm. bread, 2 gm. salt mixture, 500 c.c. milk											
2/10	.....	.....	...	...	42.0	102	0.68	7,5	8,6	11.30	...	* Poik.
2/16	1,406	1,314	718	582	44.3	107	0.69	7,8	7,2	11.50	114	* Poik.

* Poikilocytosis of red blood cells.

† Salt mixture No. 185 (McCollum and Simmonds).

Table 89 is of much interest because we have control observations on the same dog. Fowler's solution has no effect whatsoever when given daily. The curve of hemoglobin regeneration shows a very slow rise from week to week, only approaching normal after five or six weeks. The normal level is never reached during the period of twelve weeks during which Fowler's solution is administered. A transfer to a period of larger food intake without Fowler's solution shows minor fluctuations from week to week during a period of four months. It is unusual for a dog to tolerate this limited diet for this length of time without any symptoms of dietary deficiency disease.

TABLE 88.—Blood Regeneration—Salt Mixture *

Dog 20-2. Bull mongrel. Male. Young adult

Date, 1919	Pigment Volume = Hb. per Cent. Times Blood Volume	Blood Volume	Plasma Volume	R. B. C. Volume	R. B. C. Hematocrit	Hb.	Color Index	R. B. C., Million	W. B. C.	Weight	Blood per Kilogram	Remarks
		C.c.	C.c.	C.c.	%	%				Kg.	C.c.	
10/27	1,878	1,498	758	732	48.9	125	0.79	7,9	7,0	17.25	87	
10/27	Diet: Bread and milk											
10/28	Bled 375 c.c.											
10/29	Bled 375 c.c. No distress											
10/31	866	1,111	760	334	30.1	78	0.93	4,2	21,6	16.35	68	
11/ 1	Bled 278 c.c.											
11/ 3	726	1,052	762	285	27.1	69	0.96	3,6	14.8	16.20	65	
11/ 3	Diet: 200 gm. bread; 500 c.c. milk, 2 gm. salt mixture*											
11/13	1,188	1,239	743	483	39.0	96	0.96	5,0	12,4	16.35	76	
11/20	1.430	1,400	788	595	42.6	102	0.80	6,4	12,2	15.75	89	† Slight
11/22	Diet: 150 gm. bread, 500 c.c. milk, 2 gm. salt mixture*											
11/26	1,285	1,377	795	574	41.7	93	0.66	7,0	6,0	15.50	89	† Slight
12/ 3	1,312	1,385	795	578	41.7	95	0.68	6,9	17,0	15.10	92	† Slight
12/10	1,502	1,386	757	616	44.4	108	0.80	6,8	11,4	14.95	93	
12/16	1,252	1,318	800	505	38.3	95	0 74	6,4	6,4	14.00	94	† Poik.‡

* Salt mixture No. 185 (McCollum and Simmonds).

† Poikilocytosis of red blood cells.

‡ Distemper and dietary deficiency disease. Recovered.

Control observations on this same dog (Table 60[8]) show the favorable influence of a diet of cooked liver under similar conditions. Hemoglobin regeneration is complete within three weeks. This gives a striking example of the beneficial effect of certain diet factors as contrasted with certain drugs.

Table 90 shows the negative effect of Fowler's solution on the new hemoglobin regeneration. The drug is given over a period of twelve weeks. The first week shows a sharp rise which is common in such experiments. Thereafter little increase is noted from week to week. The pigment volume remains unchanged after the first three weeks. Compare other similar experiments on this same dog cited above, (Tables 85 and 88).

Following the long period of bread and milk diet, plus Fowler's solution, the dog was given a liberal mixed diet. As is not infrequent after long periods of limited diet, we note an actual fall in hemoglobin which in this case is due to the great gain in weight and increase in plasma volume. The following two weeks show continued gain in weight, increase in plasma volume, hemoglobin and red hematocrit. This illustrates the favorable reaction, even following a prolonged limited diet period, which follows a mixed diet rich in meat.

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[8] Whipple, Robscheit and Hooper: Am. J. Physiol. **53**:251. 1920.

TABLE 89.—BLOOD REGENERATION—FOWLER'S SOLUTION

Dog 19-83. Bull mongrel. Male. Adult

Date, 1920	Pigment Volume = Hb. per Cent. Times Blood Volume	Blood Volume	Plasma Volume	R. B. C. Volume	R. B. C. Hematocrit	Hb.	Color Index	R. B. C., Million	W. B. C.	Weight	Blood per Kilogram	Remarks
		C.c.	C.c.	C.c.	%	%				Kg.	C.c.	
3/ 4	1,985	1,470	578	873	59.4	135	0.61	11,8	8,2	13.20	111	
3/ 4	Diet: Bread and milk											
3/ 5	Bled 368 c.c.											
3/ 6	Bled 368 c.c.											
3/ 8	.....	.....	...	...	34.0	...	....	...	....	.....	...	
3/ 8	Bled 325 c.c.											
3/10	780	1,207	850	345	28.6	64	0.77	4,2	14,2	12.75	95	* Slight
3/10	Diet: 150 gm. bread, 500 c.c. milk, 5 drops Fowler's solution											
3/17	1,114	1,475	944	516	35.0	76	0.73	5,2	12,4	12.75	116	* Slight
3/25	1,110	1,297	766	519	40.0	86	0.68	6,3	11,6	12.25	106	* Slight
4/ 1	1,409	1,505	828	662	44.0	94	0.65	7,2	10,4	12.00	125	* Slight
4/ 7	1,450	1,470	765	692	47.0	99	0.61	8,1	6,2	12.05	122	* Slight
4/15	1,485	1,470	771	685	46.6	101	0.63	7,9	6,4	11.95	123	* Slight
4/22	1,510	1,398	692	692	49.5	108	0.65	8,3	5,6	11.60	120	* Slight
4/29	1,505	1,468	745	708	48.2	102	0.59	8,7	4,8	11.55	127	* Slight
5/ 6	1,345	1,345	700	632	47.0	100	0.60	8,3	5,2	11.55	116	* Poik.+
5/14	1,340	1,390	721	657	47.2	96	0.61	7,9	6,4	11.00	126	* Poik.+
5/21	1,204	1,276	657	606	47.5	94	0.61	7,7	6,0	10.85	117	* Slight
5/28	1,419	1,375	702	667	48.5	103	0.61	8,5	5,2	10.70	128	* Slight
5/29	Diet: 250 gm. bread, 500 c.c. milk. Fowler's solution discontinued											
6/ 4	.....	.....	...	...	44.8	102	....	...	....	.....	...	
6/11	1,305	1,393	743	587	43.7	93	0.57	8,2	6,4	11.70	114	* Slight
6/18	1,307	1,330	749	571	42.9	98	0.55	8,9	5,4	11.80	113	* Poik.
6/26	1,508	1,445	778	651	45.2	104	0.56	9,3	6,8	12.25	118	* Poik.
7/ 9	1,325	1,368	803	551	40.3	97	0.64	7,6	7,2	12.05	113	* Poik.
7/28	1,692	1,500	775	710	47.4	108	0.72	7,8	6,4	12.40	121	* Slight
8/18	1,960	1,621	803	803	49.5	121	0.79	7,6	6,0	12.85	126	* Poik.
8/30	1,705	1,505	780	711	47.2	113	0.74	7,6	5,8	12.10	124	* Poik.
9/ 8	1,630	1,538	854	669	43.5	106	0.68	7,8	6,2	12.60	122	* Poik.+
9/22	1,550	1,550	894	640	41.3	100	0.69	7,2	7,2	12.75	121	* Poik.

* Poikilocytosis of red blood cells.

Table 91 shows that another arsenical, sodium cacodylate, is inert under these conditions. The curve of blood regeneration rises slowly and uniformly during 11 weeks but never returns to normal. This reaction is typical of many recorded for bread and milk alone. A change of diet gives a prompt increase in all hemoglobin figures and the normal level is reached in three weeks. Under such conditions this reaction to meat may not be as prompt, but it illustrates this favorable reaction in contrast to the negative influence of this drug, sodium cacodylate.

TABLE 90.—Blood Regeneration—Fowler's Solution

Dog 20-2. Bull mongrel. Male. Young adult

Date, 1920	Pigment Volume = Hb. per Cent. Times Blood Volume	Blood Volume	Plasma Volume	R. B. C. Volume	R. B. C. Hematocrit	Hb.	Color Index	R. B. C., Million	W. B. C.	Weight	Blood per Kilogram	Remarks
		C.c.	C.c.	C.c.	%	%				Kg.	C.c.	
3/ 4	2,282	1,915	882	1,013	52.9	119	0.60	10,0	13,4	17.65	108	
3/ 4	Diet: Bread and milk											
3/ 5	Bled 480 c.c.											
3/ 6	Bled 480 c.c.											
3/ 8	.....	.....	...	...	28.6	...	....	...	....	.....	...	
3/ 8	Bled 350 c.c.											
3/10	662	1,350	1,047	289	21.4	49	0.84	2,9	33,4	17.00	79	
3/10	Diet: 150 gm. bread, 500 c.c. milk, 2 drops of Fowler's solution											
3/17	1,180	1,370	803	554	40.5	86	0.81	5,3	7,0	15.85	86	
3/18	Diet: 150 gm. bread, 500 c.c. milk, 5 drops of Fowler's solution											
3/25	1,243	1,495	904	575	38.5	83	0.78	5,3	6,8	15.60	96	
4/ 1	1,410	1,535	857	695	45.3	92	0.71	6,5	7,2	15.00	102	
4/ 7	1,660	1,680	885	786	46.8	99	0.73	6,8	5,4	15.05	112	
4/15	1,750	1,730	912	815	47.1	101	0.73	6,9	5,6	15.05	115	
4/22	1,715	1,715	850	850	49.5	100	0.60	8,4	6,6	14.85	115	
4/29	1,695	1,740	916	808	46.4	97	0.65	7,5	7,0	14.65	119	* Slight
5/ 6	1,530	1,570	820	734	46.8	98	0.60	8,1	6,8	14.55	108	* Poik.+
5/14	1,565	1,565	820	738	47.1	100	0.63	8,0	5,6	13.75	114	* Poik.+
5/21	1,387	1,547	857	675	43.6	90	0.62	7,2	6,4	13.50	115	* Poik.
5/28	1,554	1,563	828	719	46.0	99	0.70	7,1	6,8	13.45	116	* Slight
5/28	Diet: 100 gm. bread, 300 gm. meat scraps, 500 c.c. milk. Fowler's solution discontinued											
6/ 4	.....	.....	...	...	43.9	99	....	...	....	.....	...	
6/11	1,395	1,545	875	680	44.0	90	0.57	7,9	6,6	15.75	98	* Poik.
6/11	Diet: 100 gm. bread, 450 gm. meat scraps, 500 c.c. milk											
6/18	2,148	1,865	924	924	49.5	115	0.66	8,4	10,0	16.40	114	
6/25	2,008	1,820	920	884	48.5	110	0.69	8,0	8,8	17.25	106	

* Poikilocytosis of red blood cells.

Table 92 confirms the reaction noted in the preceding Table 91. We believe there is not a bit of evidence that the sodium cacodylate has any effect upon this reaction. There is every reason to believe that an identical reaction would have been observed in this experiment had the drug not been given. Compare Table 86 above, in which a more favorable reaction is associated with the oral administration of a standard salt mixture (McCollum and Simmonds).

SUMMARY

Iron in the form of Blaud's pills is inert when given under controlled conditions in anemia periods under the conditions of these experiments (Paper V of this series [4]).

The anemia is produced by hemorrhage, and the curve of regeneration followed week by week, the dogs being maintained on a fixed diet.

Iron in the form of ferric citrate (subcutaneously) and ovoferrin (orally) has no significant effect on the curve of hemoglobin regeneration under controlled conditions.

TABLE 91.—Blood Regeneration—Sodium Cacodylate

Dog 20-5. Bull mongrel. Male. Young adult

Date, 1920	Pigment Volume = Hb. per Cent. Times Blood Volume	Blood Volume	Plasma Volume	R. B. C. Volume	R. B. C. Hematocrit	Hb.	Color Index	R. B. C., Million	W. B. C.	Weight	Blood per Kilogram	Remarks
		C.c.	C.c.	C.c.	%	%				Kg.	C.c.	
3/ 4	2,360	1,800	774	1,008	56.0	131	0.75	8,7	12,2	13.75	131	
3/ 4	Diet: Bread and milk											
3/ 5	Bled 450 c.c.											
3/ 6	Bled 450 c.c.											
3/ 8	.....	.....	...	...	27.5	...	....	...	....	.....	...	
3/ 8	Bled 300 c.c.											
3/10	520	1,170	924	234	20.0	44	0.77	2,9	13,6	13.40	87	
3/10	Diet: 150 gm. bread, 500 c.c. milk, 0.1 gm. sodium cacodylate subcutaneously											
3/17	637	1,250	946	310	24.8	51	0.77	3,3	12,6	13.25	94	
3/25	798	1,310	927	369	28.2	61	0.65	4,7	11,8	13.00	101	* Slight
4/ 1	782	1,325	922	392	29.6	59	0.54	5,5	11,2	12.90	103	* Poik.+
4/ 7	1,079	1,370	872	512	37.3	79	0.50	7,8	8,8	12.85	107	* Poik.
4/15	970	1,320	857	448	34.0	73	0.52	7,1	8,4	12.85	103	* Poik.++
4/22	1,162	1,352	803	542	40.1	86	0.45	9,6	7,6	12.45	108	* Poik.++
4/29	1,125	1,380	823	548	39.7	81	0.60	7,6	8,8	12.55	110	* Poik.
5/ 6	1,258	1,455	840	600	41.2	86	0.49	8,9	8,2	12.40	117	* Poik.−+
5/14	1,252	1,360	754	592	43.6	92	0.58	8,0	6,0	12.25	111	* Poik.+
5/21	1,225	1,385	788	584	42.2	88	0.50	8,9	6,2	12.40	112	* Poik.++
5/28	1,330	1,420	778	634	44.7	94	0.73	9,4	5,0	12.25	116	* Poik.+
5/28	Diet: 100 gm. bread, 300 gm. meat scraps; 500 c.c. milk. Sod. cacodylate discontinued											
6/ 4	.....	.....	...	...	45.0	100	....	...	....	.....	...	
6/11	1,745	1,650	868	768	46.5	106	0.58	9,1	6,0	14.10	117	* Poik.
6/11	Diet: 100 gm. bread, 450 gm. meat scraps, 500 c.c. milk											
6/18	1,998	1,745	876	852	48.8	114	0.58	9,8	17,4	14.90	117	
6/25	2,335	1,861	850	906	53.5	125	0.63	10,5	16,4	15.50	120	

* Poikilocytosis of red blood cells.

Iron forming a part of a standard salt mixture (McCollum and Simmonds) may be concerned in a somewhat favorable anemia reaction, but even this reaction is not surely related to the iron salt. At best this reaction does not compare favorably with those recorded in association with certain potent diet factors.

Iron in the form of hemoglobin (organic iron) may be concerned in the somewhat favorable reaction observed following the administration of hemoglobin orally, intraperitoneally or intravenously. But the iron may be less concerned in this reaction than the pyrrol complex (Paper V of this series[4]).

Arsenic in the form of sodium cacodylate or Fowler's solution is inert in these same anemia periods. No drug has been tested which can compare with the meat factors in stimulating a rapid regeneration of hemoglobin during these anemia periods induced by simple hemorrhage.

TABLE 92.—Blood Regeneration—Sodium Cacodylate

Dog 20-3. Bull mongrel. Male. Young adult

Date, 1920	Pigment Volume = Hb. per Cent. Times Blood Volume	Blood Volume	Plasma Volume	R. B. C. Volume	R. B. C. Hematocrit	Hb.	Color Index	R. B. C., Million	W. B. C.	Weight	Blood per Kilogram	Remarks
		C.c.	C.c.	C.c.	%	%				Kg.	C.c.	
3/ 4	2,165	1,865	838	1,010	54.2	116	0.69	8,4	13,2	16.95	110	
3/ 4	Diet: Bread and milk											
3/ 5	Bled 467 c.c.											
3/ 6	Bled 467 c.c.											
3/ 8	.....	.....	...	...	24.4	...	....	...	....	.....	...	
3/ 8	Bled 200 c.c.											
3/10	640	1,320	1,009	294	22.3	49	0.93	2,6	30,8	16.40	80	
3/10	Diet: 150 gm. bread, 500 c.c. milk, 0.1 gm. sodium cacodylate subcutaneously											
3/17	1,180	1,548	1,018	508	32.8	70	0.81	4,3	10,6	16.05	96	
3/25	1,207	1,563	923	633	40.5	83	0.78	5,3	9,4	15.00	104	* Slight
4/ 1	1,480	1,625	896	745	45.8	91	0.72	6,3	9,6	15.00	106	* Slight
4/ 7	1,580	1,602	850	726	45.3	99	0.81	6,1	8,2	14.85	108	* Slight
4/15	1,755	1,675	874	796	47.5	105	0.81	6,5	7,8	14.20	118	* Slight
4/22	1,775	1,715	864	840	49.0	103	0.71	7,3	5,6	14.25	120	* Slight
4/29	1,788	1,700	808	876	51.5	105	0.65	8,1	6,8	13.95	122	* Slight
5/ 6	1,815	1,760	904	834	47.4	103	0.72	7,2	6,4	13.80	127	* Poik.
5/14	1,690	1,497	752	732	48.9	113	0.86	6,6	10,6	13.35	112	* Slight
5/21	1 317	1,480	829	636	43.0	89	0.74	6,0	9,4	13.05	113	* Poik.
5/28	1,310	1,455	837	612	42.0	90	0.76	5,9	8,4	12.55	116	* Slight
5/29	Diet: 100 gm. bread, 300 gm. meat scraps, 500 c.c. milk. No sodium cacodylate											
6/ 4	.....	.....	...	...	43.6	101	....	...	....	.....	...	
6/11	1,607	1,630	848	765	47.0	99	0.70	7,0	7,2	14.20	114	
6/11	Diet: 100 gm. bread, 450 gm. meat scraps, 500 c.c. milk											
6/18	1,852	1,657	826	816	49.2	112	0.67	8,4	12,4	14.65	113	
6/25	1,782	1,647	851	784	47.6	108	0.68	7,9	10,6	15.75	105	

* Poikilocytosis of red blood cells.

These carefully controlled experiments give no support to the time honored custom of administering iron and certain other drugs in conditions of simple anemia. The burden of proof now rests with those who claim that any given drug is potent under such conditions. In such experiments or clinical observations the diet factors must be carefully studied. We believe it will be of much clinical importance to record accurate observations in human cases, giving particular attention to a variety of diet factors.

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9. McCollum and Simmonds: J. Biol. Chem. **33**:63, 1918.

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AMERICAN MEDICAL ASSOCIATION
FIVE HUNDRED AND THIRTY-FIVE NORTH DEARBORN STREET
CHICAGO

I. Liver Regeneration Following Chloroform Injury as Influenced by the Feeding of Casein or Gelatin

N. C. DAVIS
AND
G. H. WHIPPLE, M.D.
SAN FRANCISCO

I. LIVER REGENERATION FOLLOWING CHLOROFORM INJURY AS INFLUENCED BY THE FEEDING OF CASEIN OR GELATIN *

N. C. DAVIS † AND G. H. WHIPPLE, M.D.
SAN FRANCISCO

This paper continues the investigation of liver cell repair as influenced by diet factors. Other papers [1] show the influence of meat, fat and carbohydrate feeding on the reconstruction of liver cells. It has been pointed out that a uniform central liver necrosis involving half or more of the liver cells can be readily produced by chloroform anesthesia or chloroform injection following a standard fasting period. Under favorable diet conditions this liver necrosis may be completely repaired by the formation of new liver cells in a period of from seven to ten days. The solution or autolysis and removal of the dead liver cells precedes the construction of new liver cells. The construction of this great mass of liver cells is a truly remarkable reaction which is worthy of much study. This speed of growth or reproduction far exceeds the new growth seen in the most malignant neoplasms or even growing embryonic tissues. It seemed highly desirable to study this new formation of specialized cells to determine the factors which may control, favor or hamper the cell reconstruction.

It is very significant that abundant carbohydrate feeding (cane sugar) is quite favorable to rapid liver cell repair and complete cell repair may be attained without the intake of any nitrogenous material. This shows very careful conservation of nitrogenous fractions in the body and proves that sugar may at times act directly by conservation of protein split products in its protein sparing action.

Glycogen is so intimately concerned in the life cycle of the liver cell that possibly sugar feeding has greater influence for the rapid development of liver cells than of other body cells. Fat apparently is not concerned in this liver cell repair and in the diet under such conditions seems to be inert, although furnishing energy requirements.

The repair and construction of red blood cells have been studied in this laboratory [2] and stand in marked contrast to the liver cell construction. Carbohydrate has a minimal influence on the red cell con-

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* From the George Williams Hooper Foundation for Medical Research, University of California Medical School.

† University of California Fellowship.

[1] Davis, N. C.; Hall, C. C., and Whipple, G. H.: Arch. Int. Med. **23**:689 (June) 1919. Davis, N. C., and Whipple, G. H.: ibid. **23**:711 (June) 1919.

[2] Whipple, G. H.; Hooper, C. W., and Robscheit, F. S.: Am. J. Physiol. **53**:167, 1920.

struction as contrasted with meat proteins, for example. This indicates wide individual variation in the repair or reconstruction complex of various body cells.

We hope to study the influence of several simple proteins and protein split products upon this standard repair of liver cells. At this time we wish to report in detail the experiments dealing with two common proteins, one of which (casein) is a complete protein and the other (gelatin) an incomplete protein.

Casein may be considered a representative of the "complete proteins" (Abderhalden, Fischer, and others). The amino-acid glycocoll, which is absent, is known to be synthesized in animal metabolism when needed. The content of cystine is relatively low. Gelatin[3] is quite high in glycocoll, but lacks the important amino-acids tyrosin, tryptophan and cystin, and is, therefore, an "incomplete protein." Both casein and gelatin are readily obtained commercially in a comparatively pure condition.

It is interesting to compare the assistance given by such different substances in the rapid repair of body tissue. Obviously our short feeding experiments of seven to ten days are not comparable with the long continued growth and general maintenance experiments.

METHOD

The routine of our regeneration experiments has been described elsewhere.[1] In brief: The animal is starved for a short period; a liver injury is obtained either by chloroform anesthesia or by subcutaneous injection of chloroform; feeding is begun the day after administration of the drug; a biopsy specimen for microscopic examination is removed from the liver on the second day (this shows the initial injury, with practically no repair); feeding is continued for any desired period following operation, usually about seven days; a liver specimen is then secured either by another operation or by sacrifice of the animal (this shows the amount of regeneration which has taken place in the liver parenchyma). An animal may frequently be used for two or more experiments.

Dogs seldom eat pure gelatin or pure casein from their food pans in any considerable amounts. It has been found satisfactory, though somewhat laborous, to make a thin casein mush in warm water and feed it to the animals by spoon. Gelatin is always given in warm solution by stomach tube. A suitable amount of salt is given with both substances. The animals will often retain their food better if the desired ration is given in divided doses throughout the day.

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[3] Fischer, E.; Levene, P. A., and Aders, R. H.: Ztschr. f. physiol. Chem. **35**:70, 1902. Levene, P. A., and Beatty, W. A.: Ibid. **49**:252, 1906.

EXPERIMENTAL OBSERVATIONS

In the accompanying table are summarized the results of feeding experiments with casein and gelatin. Protocols follow.

Liver Repair Influenced by Feeding of Casein and Gelatin

Experiment	Liver Injury	Feeding, Days	Average Daily Amount Food, Gm.	Liver Repair	Remarks
Casein No. 1 Dog 20-41	55-60% necrosis; trace of fat	8	100	20% unrepaired; no fat	Some vomiting on 2d day; developed distemper; killed
No. 2 Dog 20-66	60-65% necrosis; slight trace of fat	9	120	15% unrepaired	Vomiting on 2d day; bright and active
No. 4 Dog 20-111	50-55% necrosis; fat moderate	7	100	20-25% unregenerated; fat heavy around central veins	Vomiting on 3d day; wound broke down; killed
No. 5 Dog 21-18	30-35% necrosis; trace of fat	9	100	Regeneration practically complete	No vomiting; maintained weight
No. 8 Dog 21-26	45-50% necrosis; fat heavy to lobule peripheries	9	60	10-15% unregenerated; fat moderate	No vomiting; died under anesthesia at final operation; necropsy negative
Gelatin No.3 Dog 20-66	60-65% necrosis; slight trace of fat	9	68	15% unrepaired; slightest possible trace of fat	Vomiting on 4th day; bright and active
No. 6 Dog 21-18	60-65% necrosis; fat heavy to lobule peripheries	9	100	Regeneration practically complete; heavy trace of fat in central cells	Vomiting on 3d day; weak toward end of experiment
No. 7 Dog 21-26	60-65% necrosis; fat heavy to lobule peripheries	9	60	10% unregenerated; fat moderate	Vomiting on 8th day; bright and active
No. 9 Dog 20-77	20-30% necrosis; severe injury up to 40%; slight scattering of fat	9	50	Regeneration practically complete; slightest possible trace of fat	Vomiting on 2d day; in good condition throughout
No. 10 Dog 20-108	60% necrosis	9	65	10-15% unregenerated	No vomiting; died under anesthesia at 2d operation; kidney sections show slight nephritis

Experiment 2.—*Casein Feeding.* Dog 20-66. White mongrel male.

Jan. 8, 1920. Weight, 21.12 pounds. Just recovered from distemper; in good condition. Isolated for starvation after daily feeding.

January 9, 10 and 11. No food.

January 12. Weight, 19.5 pounds. Active. Chloroform anesthesia, 1 hour (5:10 to 6:10 p. m.).

January 13. Weight, 19.5 pounds. Active. Left 125 gm. casein, and salt, and a little beef extract (all moistened) in cage.

January 14. Weight, 19 pounds. Active. Ate about 75 gm. casein left in cage. Piece of liver removed at 2:30 p. m. Vomited later. Left a little casein in cage—no forced feeding.

January 15. Weight, 18.5 pounds. Bright and active. 120 gm. casein by spoon feeding.

January 16. Weight, 19 pounds. Bright and active. 125 gm. casein by spoon feeding.

January 17. Weight, 18.7 pounds. Bright and active. 125 gm. casein by spoon feeding.

January 18. Weight, 18.7 pounds. Bright and active. 125 gm. casein by spoon feeding.

January 19. Weight, 18.4 pounds. Bright and active. 125 gm. casein by spoon feeding.

January 21. Piece of liver removed at 2: 30 p. m.

January 28. Wounds almost well. In excellent condition.

Sections: First operation, from 60 to 65 per cent. necrosis. Slight trace of fat.

Sections: Second operation, 15 per cent. unrepaired.

Experiment 5.—*Casein Feeding.* Dog 21-18. Young brindle mongrel, male.

Sept. 14, 1920. Weight, 25.5 pounds. In good condition. Old keratitis and staphyloma of right eye suggest previous distemper infection. Isolated after daily feeding: fasting begun.

September 15, 16 and 17. No food.

September 19. Weight, 23.8 pounds. Chloroform anesthesia for forty-five minutes (3 to 3: 45 p. m.). Gave 10 mg. sodium cyanid in 100 c.c. physiologic sodium chlorid solution intravenously during thirty-five minutes of anesthesia.

September 18. Weight, 23.3 pounds. A little dull. Gave 100 gm. casein and salt by spoon feeding.

September 20. Weight, 23.5 pounds. A little dull. Gave 100 gm. casein and salt as before. Piece of liver removed at 3 p. m. Liver looks injured in gross.

September 21. Weight, 23.25 pounds. Dull. Nose shows slight discharge. 100 gm. casein.

September 22. Weight, 23.4 pounds. Dull. Nose shows slight discharge. 100 gm. casein.

September 23. Weight, 23.4 pounds. Wound infected, but not broken down. 100 gm. casein.

September 24. Weight, 23.7 pounds. Nose condition clearing up. 100 gm. casein.

September 25. Weight, 23.7 pounds. No nasal discharge. Thin purulent oozing from wound. 100 gm. casein.

September 26. Weight, 23.8 pounds. Constipation probably accounts for some of gain in weight. 100 gm. casein.

September 27. Weight, 23.7 pounds. Wound superficially open. 100 gm. casein. Operation at 2: 30 p. m. Small piece of liver removed.

Sections: First operation, from 30 to 35 per cent. necrosis; trace of fat.

Sections: Second operation, almost complete regeneration; less than 10 per cent. not filled out. Slightest possible trace of fat.

Experiment 6.—*Gelatin Feeding.* Dog 21-18. Young brindle mongrel, male.

Oct. 19, 1920. Weight, 30.7 pounds. Former operative sites in good condition. Isolated after daily feeding; fasting begun.

October 20, 21 and 22. No food.

October 23. Weight, 27.5 pounds. Active and in good condition. Chloroform anesthesia for forty-five minutes (3: 40 to 4: 25 p. m.). 100 c.c. physiologic sodium chlorid solution intravenously during anesthesia.

October 24. Weight, 27 pounds. Active. 100 gm. gelatin and salt in 300 c.c. water by stomach tube.

October 25. Weight, 25.5 pounds. Active. No vomiting. 100 gm. gelatin given in divided doses. Piece of liver removed at 2: 30 p. m.

October 26. Weight, 25.5 pounds. Bright and active. 100 gm. gelatin in divided doses (partly vomited).

October 27. Weight, 25.4 pounds. Active. 100 gm. gelatin as before. No vomiting.

October 28. Weight, 25 pounds. Rather quiet. 100 gm. gelatin as before. No vomiting.

October 29. Weight, 25.1 pounds. Quiet and somewhat weak. 100 gm. gelatin as before. No vomiting.

October 30. Weight, 25.25 pounds. Rather weak. Removed stitches yesterday evening and wound has opened because of infection. 100 gm. gelatin in divided doses. No vomiting.

October 31. Weight, 24.75 pounds. Seems bright, but not active. 100 gm. gelatin at one feeding. No vomiting.

November 1. Weight, 24.5 pounds. Inactive. 100 gm. gelatin at one feeding. No vomiting. Piece of liver removed at 3:30 p. m.

Sections: Third operation, from 60 to 65 per cent. necrosis; fat heavy to lobule peripheries.

Sections: Fourth operation, almost complete filling in of lobules (less than 10 per cent. unregenerated); lobule centers show heavy trace of fat.

Experiment 7.—*Gelatin Feeding.* Dog 21-26. Young, long-haired mongrel, female.

Oct. 5, 1920. Weight, 18.8 pounds. In good condition: active. Isolated after daily feeding; fasting begun.

October 6, 7 and 8. No food.

October 9. Weight, 16.9 pounds (7.7 kilos). Active. Chloroform anesthesia for forty-five minutes (3:35 to 4:20 p. m.). During forty minutes of this time 260 c.c. of 1 per cent. sodium carbonate in hypertonic saline solution (1.4 per cent.) were given intravenously (approximately 34 c.c. per kilo).

October 10. Weight, 16.25 pounds. Bright and active. Gave 60 gm. gelatin and salt in 200 c.c. water by stomach tube.

October 11. Weight, 16.5 pounds. Active. No vomiting yesterday. 60 gm. gelatin. Piece of liver removed at 4 p. m. Liver looks fatty.

October 12. Weight, 16.4 pounds. Quiet. 60 gm. gelatin by stomach tube (lost a little).

October 13. Weight, 15.75 pounds. More active today. 60 gm. gelatin. No vomiting.

October 14. Weight, 15.5 pounds. Rather quiet. 60 gm. gelatin in divided doses. No vomiting.

October 15. Weight, 15.4 pounds. Quite active. 30 gm. gelatin in morning, 60 gm. in evening.

October 17. Weight, 14.5 pounds. Active. 75 gm. gelatin. Wound open a little.

October 18. Weight, 14.6 pounds. Bright and active. 75 gm. gelatin (Vomited some last night.) Piece of liver removed at 2:30 p.m. Encountered a little peritoneal fluid, slightly cloudy.

Sections: First operation: 45 to 50 per cent. necrosis; fat heavy to lobule peripheries.

Sections: Second operation: about 10 per cent. unregenerated; fat moderate.

Experiment 8.—*Casein Feeding.* Dog 21-26. Young, long-haired mongrel, female.

November 16, 1920. Weight, 18.9 pounds. In good condition. Dog had a touch of distemper since last used, but has recovered. Isolated after daily feeding; fasting begun.

November 17, 18 and 19. No food.

November 20. Weight, 16.6 pounds (7.55 kilos). In good condition, active. Chloroform anesthesia for forty-five minutes (2:05 to 2:50 p. m.). During forty minutes of this period 260 c.c. hypertonic saline solution (1.4 per cent.) was given intravenously (34 + c.c. per kilo).

November 21. Weight, 16 pounds. Bright and active. 60 gm. casein and salt by spoon feeding.

November 22. Weight, 16.5 pounds. Bright and active. 60 gm. casein and salt by spoon feeding. Some casein was vomited yesterday . Piece of liver removed at 12 noon; fatty and friable.

November 23. Weight, 16.25 pounds. Somewhat weak. There appears to have been some oozing from wound. 60 gm. casein in divided doses.

November 24. Weight, 16 pounds. Bright and active. 60 gm. casein in divided doses.

November 25. Weight, 16 pounds. Bright and active. 60 gm. casein at one feeding.

November 26. Weight, 16.1 pounds. Bright and active. 60 gm. casein at one feeding.

November 27. Weight, 15.9 pounds. Bright and active. 60 gm. casein at one feeding.

November 28. Weight, 15.6 pounds. Bright and active. 60 gm. casein at one feeding. Wound healing nicely.

November 29. Weight, 15.4 pounds. Bright and active. 60 gm. casein at one feeding. Operation in afternoon; dog died under anesthesia. Necropsy essentially negative.

Sections: Third operation: from 45 to 50 per cent. necrosis; fat heavy to lobule peripheries.

Sections: Necropsy: from 10 to 15 per cent. unregenerated; fat moderate.

EXPERIMENT 9.—*Gelatin Feeding.* Dog 20-77. Young female terrier.

Feb. 21, 1920. Weight, 11.5 pounds. Active and in good condition. Isolated after daily feeding; fasting begun.

February 22, 23, 24 and 25. No food.

February 25. Weight, 10 pounds. Active. Chloroform anesthesia one hour and ten minutes (3:20 to 4:30 p. m.).

February 26. Weight, 9.5 pounds. Active. 50 gm. gelatin in 200 c.c. water by stomach tube.

February 27. Weight, 9.5 pounds. Active. 50 gm. gelatin in 200 c.c. water by stomach tube. Food partly vomited. Piece of liver removed at 10:30 a. m.

February 28. Weight, 9.5 pounds. Active. 50 gm. gelatin by stomach tube.

February 29. Weight, 9.1 pounds. Active. 50 gm. gelatin by stomach tube.

March 2. Weight, 8.75 pounds. Active. 50 gm. gelatin by stomach tube.

March 2. Weight, 8.6 pounds. Active. 50 gm. gelatin by stomach tube.

March 3. Weight, 8.4 pounds. Active. 50 gm. gelatin by stomach tube. Operation wound opening slightly.

March 4. Weight, 8.2 pounds. Rather quiet. 50 gm. gelatin by stomach tube.

March 5. Weight, 8.2 pounds. Rather quiet. 50 gm. gelatin by stomach tube. 3:30 p. m., removed piece of liver.

Sections: Second operation: from 25 to 30 per cent. necrosis, injured up to 40 per cent, with scattering of fat.

Sections: Third operation; slightest possible trace of fat; repair almost complete.

The following experiments were undertaken with the idea of supplementing a sugar diet with casein. The protein was digested with pancreatic extract so that it became soluble. Since the digest was very nauseating, an attempt was made in one experiment to give it by the intraperitoneal route. In addition to the animals in Experiments 12 and 13, another dog was given sugar and casein digest by stomach tube. This dog died on the sixth day after chloroforming with very toxic symptoms and autopsy showed less than one-half of the liver injury repaired.

It is clear that this casein digest amino-acid mixture was much more toxic to a dog poisoned by chloroform than to a normal animal. The significance of this observation is not apparent but further investigation of this point should be made.

EXPERIMENT 11.—*Casein Digest Intraperitoneally. Control.* Dog 20-78. Long-haired mongrel, male.

Feb. 4, 1920. Given 5 gm. casein digest dissolved in 35 c.c. physiologic sodium chlorid solution, intraperitoneally. No reaction.

February 5 and 6. Dog clinically as usual.

February 7. 10 gm. casein digest intraperitoneally. No reaction.

Feb. 8 and 9. As well as usual.

February 10. Sacrificed. Necropsy negative.

EXPERIMENT 12.—*Sugar Feeding; Casein Digest Intraperitoneally.* Dog 20-64. Large, black spaniel, female.

Feb. 7, 1920. Weight, 33.75 pounds. In excellent condition. Isolated after daily feeding; fasting begun.

February 8, 9 and 10. No food.

February 11. Weight, 29.6 pounds. Active. Chloroform anesthesia for one hour and five minutes (3:30 to 4:35 p. m.).

February 12. Weight, 29.2 pounds. Bright and active. 100 gm. cane sugar and kaolin by stomach tube.

February 13. Weight, 28.6 pounds. Somewhat dull and weak. Food as yesterday. Piece of liver removed at 3:45 p. m.; gross appearance of severe injury. (Sections show from 50 to 55 per cent. necrosis.)

February 14. Weight, 29 pounds. Dog is quite sick. Pulse rapid and weak; temperature subnormal. No jaundice or hemorrhages; 100 gm. cane sugar by stomach tube; 5 gm. protein digest intraperitoneally in 25 c.c. water. All intraperitoneal injections given under light ether anesthesia.

February 15. Weight, 29.3 pounds. Weak, but not so sick as yesterday. Sclera jaundiced. 100 gm. sugar by stomach tube; 5 gm. protein digest in 20 c.c. fluid intraperitoneally.

February 16. Weight, 28.3 pounds. Still weak. 100 gm. sugar by stomach tube; 10 gm. protein digest in 45 c.c. fluid intraperitoneally.

Feb. 17. Weight, 28 pounds. Sugar as before; 10 gm. protein digest in 30 c.c. fluid intraperitoneally.

February 18. Weight, 27.6 pounds. 150 gm. sugar by stomach tube; 15 gm. protein digest in 45 c.c. water intraperitoneally.

February 19. Weight, 27.25 pounds. Sugar and protein digest as yesterday. Weak.

February 20. Weight, 25.1 pounds. Sugar and protein digest as yesterday. Vomited a little. Very weak. Great quantities of thin, reddish-brown diarrhea. Died about noon.

Necropsy Report.—All tissues intensely jaundiced Some hemorrhage into abdominal wall, especially around operative site and points of injection. Peritoneal cavity contains some fluid, perhaps largely from injection in morning; fluid looks blood tinged. Peritoneal surfaces and mesentery are engorged and purplish; there are occasional fibrin deposits; a loop of intestine is adhered to anterior wall on left side. Fat overlying pericardium appears hemorrhagic. Heart negative. Blood is massively clotted.

Lungs are mottled light and dark (mostly posterior), but there seem to be no hemorrhages or consolidation.

Liver, in addition to jaundice, is extremely mottled, suggesting great lack of repair. There are a number of small antemortem clots throughout the parenchyma.

Spleen shows extensive hemorrhages.

Kidneys: dark; cortices somewhat swollen and cloudy, suggesting parenchymatous degeneration.

Mucosa of gastro-intestinal tract and bladder negative.

Microscopic Report.—Lungs: hemorrhagic exudate; congestion. Spleen and lymph nodes: hemorrhages and necrosis. Liver: from 10 to 15 per cent. not repaired. Kidneys: cloudy swelling.

EXPERIMENT 13.—*Sugar and Casein Digest Feeding.* Dog 20-76. Black mongrel, female.

April 28, 1920. Weight, 14.75 pounds. In good condition (three previous experiments). Isolated after daily feeding; fasting begun.

April 29 and 30. No food.

May 1. Weight, 13.25 pounds (6.023 kilos). Dog in good condition. Gave 1.27 c.c. chloroform (0.21 c.c. per kilo) plus twice its volume liquid petrolatum subcutaneously.

May 2. Weight, 13.1 pounds. Bright and active. Gave by stomach tube 50 gm. cane sugar, 15 gm. protein digest and a little kaolin.

May 3.—Weight, 12.9 pounds. Vomited some yesterday. Food as yesterday except 5 gm. less amino-acids. Vomited some about one and a half hours after feeding. Operation at 2:30 p. m. Cut out one of preceding operative scars; no difficulties. Piece of liver removed.

May 4. Weight, 12.6 pounds. Active. Fed 100 gm. cane sugar and 15 gm. protein digest in divided doses, by stomach tube.

May 5. Weight, 12.3 pounds. Active. Food as yesterday. Some vomiting in morning.

May 6. Weight, 12.1 pounds. Active. Food as yesterday. Some vomited in morning. Put dog in metabolism cage; to receive less water.

May 7. Weight, 12 pounds. Quiet. Food as yesterday. Not vomited.

May 8. Weight, 11.4 pounds. Quiet. Food as yesterday. Partly vomited.

May 9. Weight, 11 pounds. Quiet. Gave 50 mg. cane sugar and 15 gm. protein digest. Largely vomited.

May 10. Dog is quite weak. Operation at 11 a. m.

The value of the experiment is much decreased because of vomiting. On some days there was little or no vomiting, and when it did occur it was often late; however, probably one third or one half of food was lost. Feces were passed on three occasions during experiment, small amounts of soft, brown material.

Operation on Second Day: 45 to 50 per cent. necrosis; fat moderate.

Operation on Ninth Day: about 15 per cent. unregenerated; trace of fat.

Note: Casein digest yields only 28 mg. amino-nitrogen per gram. indicating that approximately 17.5 per cent. of mixture consists of amino-acids.

DISCUSSION

A comparison of the liver regeneration on diets of casein and gelatin shows that in this series the results have been about the same, —the superiority, if any, resting with gelatin. This means that the liver cells were not repaired entirely by amino acids coming from the gelatin, but that the latter must necessarily have been supplemented by "building stones" from endogenous katabolic waste material perhaps in part from the necrotic liver cells.

Regeneration with these isolated proteins has been equal to that with meat diets, better than that on starvation or with fat diet, and less complete than that with mixed table scraps, bread and milk, or even a pure carbohydrate diet.[1] One might infer that in the rapid regeneration of liver cell protein the conservation of metabolic end products plays a larger part than the furnishing of new protein material. Evidently, under certain conditions, energy requirements have precedence over reparative processes to the extent that incoming protein material may be stripped by de-aminization, to a point where synthesis of cell protoplasm is hampered.

Owing to the tendency to vomiting, it has usually been necessary to give less of gelatin than of casein per day. Loss of weight has been less marked in the case of casein feeding.

In experiment 7, carbonate solution was given intravenously during chloroform anesthesia, while in Experiment 8 on the same dog only saline solution was injected, yet the injuries were identical. Whether or not chloroform causes an acidosis, and this in turn is relieved by alkaline therapy, has no bearing on our work—carbonate solution intravenously certainly is not able to prevent in any measure the liver injury caused by chloroform.

SUMMARY

Gelatin, although an "incomplete protein," seems to be equally as efficacious as casein in the regeneration of liver parenchyma destroyed by chloroform.

After a 50 to 60 per cent. necrosis, a feeding period of nine days with either substance will restore the liver to within 10 or 15 per cent. of normal. This liver cell regeneration is comparable to that obtained with a meat diet, approaches that given by a high carbohydrate diet, is definitely less than with a full mixed diet, but greater than with a fat diet or with fasting.

The liver repair on gelatin feeding emphasizes again the important fact that the body is able to conserve certain amino acids or split products from its endogenous wastage products and recast these groups into the complex liver cell.

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CHICAGO

II. The Effect of Cyanids and of Organic Oxidizing Agents on the Liver Injury Caused by Chloroform

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II. THE EFFECT OF CYANIDS AND OF ORGANIC OXIDIZING AGENTS ON THE LIVER INJURY CAUSED BY CHLOROFORM [*]

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SAN FRANCISCO

The series of experiments here recorded were undertaken with the hope of throwing some light on the etiology of chloroform liver necrosis. Should this liver injury be caused by a reduction of tissue oxidation, it is conceivable that the cyanids, which apparently act by preventing oxidation, might increase the damage to be expected from chloroform if the two drugs were given simultaneously. On the other hand, iodozo- and iodoxy-benzoic acids and their sodium salts, which readily give up oxygen to the tissues, might lessen the expected injury.[1]

We have already reported a few experiments in which potassium cyanid was administered intravenously, either alone or in conjunction with prolonged chloroform anesthesia.[2] Although large doses of the drug itself produce an extensive fatty alteration in the liver parenchyma, smaller amounts, given during the administration of chloroform, affect the liver changes produced by the anesthetic very little, if at all.

Experimental work by Verworn [3] tends to show that the depression caused by narcotics is really an asphyxiation. His hypothetical suggestion is that the oxygen carriers are unable to supply oxygen to the tissues, perhaps because the enzymes are of a lipoidal nature with consequent affinity for the usual anesthetics. Burge [4] reports that the oxidative enzyme catalase is greatly reduced by anesthetics, most pronouncedly by chloroform. This might be considered to support the views of Verworn. However, both the method [5] and the conclusions of Burge have frequently been questioned.[6]

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[*] From the George Williams Hooper Foundation for Medical Research, University of California Medical School.

[†] University of California Fellowship.

1. We are indebted to Professor Loevenhart of the University of Wisconsin for liberal supplies of iodozo- and iodoxy-benzoic acids, also for valuable criticisms and suggestions relative to the experiments.

2. Davis, N. C., and Whipple, G. H.: Arch. Int. Med. **23**:636 (May) 1919.

3. Verworn, Max: Harvey Lectures **7**: 1911.

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6. Becht, F. C.: Am. J. Physiol. **48**:171, 1919. Reimann, S. P., and Becker, C. A.: J. Physiol. **50**:54, 1919. Stehle, R. L.: J. Biol. Chem. **39**:403, 1919.

Buckmaster [7] has shown that at the point of anesthesia with chloroform, from 64 to 71 per cent. of the drug is held by the red corpuscles; after inhalation of 2 per cent. chloroform for three-quarters of an hour, 98.5 per cent. is found in the erythrocytes. He also demonstrated that in the first three minutes of anesthesia lung ventilation is reduced from 30 to 80 per cent.; he considered, however, that the reduction of oxygen in the blood is not due to the latter cause but to the effect of the chloroform on the corpuscles (oxygen carriers).

Graham [8] has stated that chloroform is one of a group of substances whose effect on organs is like that of asphyxiation. He surmised that these drugs dissociate in the body, yielding a molecule with bivalent or unsaturated carbon which eagerly seizes oxygen. However, in a recent article [9] he has dismissed this theory. Buckmaster [7] has reported that he could not detect the formation of carbon monoxid from chloroform in the body.

Wells [10] has suggested that chloroform may first unite with the cell lipoids, perhaps physically, then poison the protoplasm by interfering with oxidative or synthetic functions.

The standard textbooks of pharmacology discuss the cyanids quite fully; Evans' [11] article on "Cyanid Anoxemia" is a good recent contribution. Work seems to show that the cyanids prevent oxidations in the tissues themselves, whether by action on protoplasm directly or by inhibition or destruction of intermediary enzymes is not quite clear. The oxygen carrying power of the blood is not impaired.

Loevenhart and his co-workers have for many years been interested in physiologic oxidations. They have been able to produce central necrosis in rabbits' livers by suboxidation, the animals being left in an atmosphere of low oxygen tension for several days at a time.[12] Loevenhart and Grove,[13] also Arkin [14] have studied the preparation of iodoxy-benzoic and iodozo-benzoic acids. Amberg,[15] Loevenhart,[16] Arkin,[17] and others have studied the effect of these acids and their sodium salts on inflammatory reactions, and the antagonism to cyanids. It has been found that cyanids intensify and organic oxidizing agents diminish inflammatory processes, such as the mustard oil reaction and the intracutaneous reaction in serum sensitization; these results seem to be due to the effect on bodily oxidations and reductions. Arkin [18] tested the bactericidal property of sodium iodoxy-benzoate and found a definite relationship between this and its oxidizing power. Arkin and Fink [19] also studied the effect of this sodium salt on catalase in the blood and tissues; they found that catalase normally varies immensely and that the values after administration of iodoxy-benzoate fell within normal limits. Loevenhart and associates [20] have recommended the therapeutic use of sodium cyanid as a respiratory stimulant. Loevenhart and Eyster [21] found that iodozo- and iodoxy-benzoates were unable to replace molecular oxygen for the maintenance of activity in the isolated mammalian heart. These oxidizing agents were reduced, however, and caused the heart to develop rigor, probably because of the oxidation of some substance in its musculature. Iodozo- and iodoxy-benzoates apparently transfer their available oxygen directly to the substance to be oxidized, without an intermediate liberation of free oxygen; certain oxidizing enzymes seem to facilitate the transfer; in certain peroxidase reactions the organic oxidizing agents act in a manner comparable to hydrogen peroxid.[16]

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METHODS

Dogs have been used as experimental animals. Rats were tried, but even the controls differed so much in reaction that the experiments are of little value; these individual variations were probably due, in part, to nutritional differences and the difficulty of imposing a fasting period to reduce the animals to a starvation base line; moreover, the immediate mortality from chloroform injection was very high. Dogs have always been starved three or four days to eliminate the effect of diet on the chloroform injury.

Squibbs' chloroform has been used for both anesthesia and injections. Chloroform anesthesia has been light; the drug was administered by the drop method. When given subcutaneously, chloroform was mixed with twice its volume of liquid petrolatum; syringe plungers

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17. Arkin, A.: J. Infect. Dis. **16**:349, 1915.

18. Arkin, A.: J. Pharmacol. & Exper. Therap. **3**:145, 1911.

19. Arkin, A., and Fink, E. B.: J. Infect. Dis. **22**:515, 1918.

20. Loevenhart, A. S.; Lorenz, W. F.; Martin, H. G., and Malone, J. Y.: **5**:21, 1913.
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21. Loevenhart, A. S., and Eyster, J. A. E.: J. Pharmacol. & Exper. Therap. **5**:21, 1913.

were removed and the substances measured directly into the barrels, first, the oil then the chloroform from a 1 c.c. pipet calibrated to hundredths; the mixtures were injected immediately after measurement. Dogs were under light ether anesthesia during the injection of chloroform and other substances. The amount of chloroform injected has not been of itself sufficient to produce unconsciousness, or even instability of equilibrium in many cases.

A liver injury produced by either method may be repeated accurately under the same experimental conditions if an intervening recovery period of three or four weeks is allowed. This possibility has been discussed in a previous communication[22] and subsequent experiments have verified our belief.

Specimens of iodozo- and iodoxy-benzoic acids were furnished by Dr. Loevenhart. His directions for preparing solutions for intravenous injections were briefly: Dissolve 1.32 gm. iodozo-benzoic acid, or 1.4 gm. iodoxy-zenzoic acid, in 20 c.c. water and 10 c.c. normal sodium hydroxid and dilute to 100 c.c. with water; solutions are to be prepared fresh just before using (intravenously). We never had occasion to use 100 c.c. at one time, hence we made up smaller amounts proportionately and by similar steps. To facilitate giving over an extended period of anesthesia these amounts were further diluted with physiologic sodium chlorid solution. When given subcutaneously, iodoxy-benzoic acid was used in relatively large amounts in small volumes of alkaline fluid. Since sodium hydroxid was the solvent, the drug actually employed was sodium iodoxy-benzoate or iodozo-benzoate, as the case might be; however, in the tables and protocols the record gives only the amount of acid corresponding to the volume of solution used. For instance, if 0.2 gm. acid was dissolved in 200 c.c. alkaline fluid, and 150 c.c. of this solution was used, the records show 0.15 gm. acid administered. Iodoxy-benzoate solution given subcutaneously was diluted with 10 per cent. acacia solution; iodozo-benzoic acid was given subcutaneously as a suspension in 10 per cent. acacia solution.

The potassium cyanid employed was a Braun-Knecht-Heimann product, from 98 to 99 per cent. pure; the sodium cyanid was supposed to be chemically pure. The cyanids were dissolved in physiologic sodium chlorid solution immediately before use.

Large amounts of fluid given intravenously have been administered by gravity method, from a funnel through a rubber tube with a glass section near the vein to act as a window, into the external jugular vein through an ordinary large syringe needle; rapidity of flow has been controlled with a screw pinch-cock.

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22. Davis, N. C., and Whipple, G. H.: Arch. Int. Med. **23**:612 (May) 1919.

In case the dogs survived, it has been our routine to operate two days after the administration of chloroform and remove a small piece of liver, either by ligating and snipping off the tip of a pointed lobe, or by inserting a mattress suture in the edge of a larger lobe and excising a wedge of tissue from the area thus deprived of blood. Operations have been performed always under ether anesthesia, and with aseptic precautions. We consider that the best time to estimate tissue injury is on the second day following the injection of the chloroform, since the necrosis is then usually well demarcated and debris is not yet in process of removal. Specimens are fixed in 10 per cent. formaldehyd solution and divided for (1) frozen sections (fat stains, etc.), and (2) paraffin embedding with subsequent hematoxylin and eosin staining. Estimates of necrosis and fatty degeneration are always based on microscopic examination of the paraffin sections.

The fact that we consider one small specimen of liver tissue as representative of the whole may appear unwarranted. Dowler and Mottram[23] studied the fat content of different parts of the liver after injection of fat into the hepatic artery, glycogen after a heavy meal of cane sugar, and fatty acids during fasting periods, and concluded that the distribution of these substances is irregular and unpredictable; however, they admit that the liver of a fasting dog gives the nearest approach to even distribution. Sérége[24] in determining urea and glycogen, and Bartlett, Corper and Long[25] in studying the distribution in the liver of fat injected in the portal vein found considerable differences between the lobes as a whole, but intralobular uniformity. Differences of distribution of these various substances have been correlated with differences in blood supply, i.e., the blood from various portal tributaries does not thoroughly mix, hence is distributed differently. On the other hand, Brissaud and Bauer,[26] also Gilbert and Villaret[27] found an equal distribution of injected gelatin throughout the liver.

When dogs have died of chloroform liver injury we have always taken sections from various parts of the liver, representing several lobes. Ignoring the single layer of liver lobules immediately beneath the capsule (which are usually severely injured), these sections have invariably shown the same picture. Consequently, we have always felt justified in basing our estimations on a biopsy specimen from one lobe only.

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EXPERIMENTAL OBSERVATIONS

Table 1 summarizes the essential details of experiments in which organic oxidizing agents were given intravenously during chloroform anesthesia. A representative protocol follows; it is scarcely necessary to add protocols for all the dogs.

Experiment 2. — *Control. Simple Starvation and Chloroform Anesthesia.* Dog 19-39, female Airdale.

Oct. 8, 1918: Weight, 26.1 pounds. Isolated before daily feeding; in good condition. Fasting begun.

October 9: Weight, 25.8 pounds; October 10: weight, 24.8 pounds. No food.

October 11: Weight, 24.5 pounds. Active and healthy. Chloroform anesthesia for one and a quarter hours (from 5:50 to 7:05 p. m.).

October 12: Weight, 23.1 pounds. Bright and active.

October 13: Weight, 22.9 pounds. Bright and active.

October 14: Weight, 22.8 pounds. Piece of liver removed at 9:30 a. m. Sections show approximately 60 per cent. necrosis (beginning resolution); fat (scattered). Mixed diet.

Chloroform Anesthesia Plus Sodium Iodoxy-Benzoate Intravenously. — Dog 19-39, female Airdale.

March 5, 1919: Weight, 33.8 pounds. Isolated before daily feeding; fasting begun; has completely recovered from previous experiments; in excellent condition.

March 6 and 7: No food.

March 8: Weight, 31.5 pounds. Active and healthy. Chloroform anesthesia for one and a quarter hours (from 2:45 to 4 p. m.). Intermittently during this period 0.21 gm. iodoxy-benzoic acid (freshly prepared in 15 c.c. alkaline solution, then diluted to 200 c.c. with physiologic sodium chlorid solution) was given intravenously. No ill effects of moment were observed either during or following the injection.

March 9: Active. Mixed food.

March 10: Active. Piece of liver removed at 1:30 p. m. Considerable bleeding occurred. Milk and mixed diet. Sections show approximately 60 per cent. necrosis with a scattering of fat in the dead tissue.

These experiments seem to indicate that iodoxy- and iodozo-benzoic acids have little, if any, protective value against liver necrosis when given intravenously. This may be due to the small amounts of the drugs so given. The intravenous solutions were administered slowly and fairly uniformly throughout anesthesia, intermittently (as in Experiment *2*), or steadily during the first forty-five or sixty minutes of anesthesia and none the last few minutes. In Experiment 1 some difficulty was encountered due to apnea during anesthesia, but the dog roused somewhat afterward; death, probably from the iodoxy-benzoate, occurred too early to determine accurately the extent of liver injury; there seemed to be some central disintegration, however. In Experiment 4 there appeared to be a definite lessening of necrosis after injection of iodozo-benzoate; in this case intravenous injection was continued steadily during the first hour of anesthesia; the liver showed one-quarter to one-third necrosis with extensive vacuolization and fat up to one-half; the uninjured lobule peripheries showed considerable

TABLE 1.—CHLOROFORM INHALATION PLUS ORGANIC OXIDIZING AGENTS INTRAVENOUSLY

Experiment Number	Dog Number	Date, 1919	Weight, Lbs.	Fasting Period, Days	Chloroform Anesthesia, Hours	Organic Oxidizing Agent	Liver Injury		Remarks
							Oxidizer Plus Chloroform	Control Chloroform	
1	19-30	Feb. 12	18.6	4	1¼	0.21 gm. iodoxybenzoic acid	?	60% necrosis	Found dead next morning; liver shows some central disintegration, necrosis not determinable; viscera congested
2	19-39	Mar. 8	31.5	4	1¼	0.21 gm. iodoxybenzoic acid	60% necrosis	60% necrosis	
3	19-74 (a)	Feb. 25	13.6	4	1¼	0.14 gm. iodoxybenzoic acid	50% necrosis	50% necrosis	
4	19-74 (b)	Apr 11	13.8	4	1¼	0.25 gm. iodozobenzoic acid	25% necrosis	50% necrosis	Glycogen appears in liver lobule peripheries, suggesting dog fed by mistake during starvation period
5	19-28	May 6	19.1	4	1¼	0.24 gm. iodozobenzoic acid	50% necrosis	50% necrosis	
6	19-135	May 24	46.5	4	1¼	0.35 gm. iodozobenzoic acid	60% necrosis	....	Operation two days following chloroform; found dead on sixth day following operation; intense jaundice; hemorrhages in viscera; slight removal of liver debris

glycogen. This glycogen content leads to the question whether or not the dog received food by mistake during the preliminary period of supposed fasting. This storage might have occurred after chloroforming and before operation, but more likely it took place before the administration of chloroform. Glycogen per se may have no protective value against chloroform, but its presence is certainly somewhat of a gage of carbohydrate assimilation, which for some reason is protective. A smaller amount of iodoxy-benzoate had no protective value for this same dog (Experiment 3). Unfortunately, there is no control on the same animal in Experiment 6, but judging from the injuries sustained by the other dogs recorded in the same table, there appears to have been no protection from the amount of iodozo-benzoic acid given.

To summarize Table 1, there seems to have been no protection against chloroform injury from intravenous administration of iodozo- and iodoxy-benzoates in five of six experiments; there seems to have been protection in one case from iodozo-benzoate, but this protection may very well have been from food given by mistake, and not from the drug.

Table 2 summarizes briefly the experiments in which chloroform was given subcutaneously with or without organic oxidizing agents and more ample summaries of certain experiments follow.

Experiment 13.—Dog 20-17, young, black mongrel, female.

Sept. 26, 1919: Weight, 18.9 pounds. Isolated before daily feeding; healthy and active; fasting begun.

September 27 and 28: No food.

September 29: Weight, 17.2 pounds (7.8 kilos). Active. Gave subcutaneously 1.17 c.c. chloroform (0.15 c.c. per kilo) plus 2.4 c.c. liquid petrolatum; temporary ether anesthesia.

September 30: Bright and active.

October 1: Weight, 16.5 pounds. Removed piece of liver at 2 p. m. Sections show about 35 per cent. necrosis with severe vacuolation and injury up to one-half; fat moderate. Recovery on mixed diet.

Experiment 21.—Dog 20-17, young black mongrel, female.

October 21: Weight, 20 pounds. In good condition and active; isolated after daily feeding; fasting begun.

October 22, 23 and 24: No food.

October 25: Weight, 18 pounds (8.18 kilos). Active. Gave subcutaneously 1.22 c.c. chloroform (0.15 c.c. per kilo) plus twice its volume liquid petrolatum, followed immediately by 1 gm. iodoxy-benzoic acid dissolved in 7 c.c. tenth-normal sodium hydroxid plus 10 c.c. of a 10 per cent. acacia solution subcutaneously. Temporary ether anesthesia.

October 26: Bright and active.

October 27: Active. Piece of liver removed at 1 p. m. Hard swelling in left lumbar quadrant at point of injection of iodoxy-benzoate. Sections show from 10 to 15 per cent. necrosis; very slight amount of fat over central half of lobules. Recovery on mixed diet. Slough at point of injection.

As will be noted from Table 2, massive doses of the organic oxidizing agents given subcutaneously apparently have protective value against chloroform injury; in some cases this is quite striking. Iodoxy-benzoic

TABLE 2.—COMPARATIVE TABLE—SUBCUTANEOUS INJECTIONS

Chloroform Alone					
Experiment Number	Dog Number	Fasting Period, Days	Chloroform C.c. per Kilo (subcutaneously)	Liver Injury	Remarks
7	20-7	4	1.00	75% necrosis	Found dead on second day
8	20-10	4	0.50	80% necrosis	Died on second day
9	20-12	4	0.20	80% necrosis	Found dead on third day
10	20-13	3	0.10	No necrosis; trace of fat	Minimal injury
11	20-13	3	0.20	75% necrosis	Found dead on third day
12	20-18	4	0.16	70% necrosis	Found dead on next day
13	20-17	4	0.15	35% necrosis; severe injury to ½	Recovered; see experiment 21 on same dog
14	20-75	3	0.15	50% (+) necrosis	Operation on second day; found dead on fourth day
15	20-76	3	0.21	60% necrosis	Recovered; see experiment 23 on same dog; second control on same dog shows only about 50% necrosis
16	20-31	4	0.18	55% necrosis	Found dead on second day; see experiment 24 on same dog
17	20-30	4	0.14	60% necrosis	Recovered; same result obtained on two occasions, once before and once after experiment 25
18	20-106	4	0.14	85% necrosis	Died on second day

Chloroform and Oxidizing Agent						
Experiment Number	Dog Number	Fasting Period, Days	Chloroform C.c. per Kilo (subcutaneously)	Organic Oxidizing Agent (subcutaneously)	Liver Injury	Remarks
19	20-38	4	0.20	Iodoxybenzoic acid 0.6 gm.	50-60% necrosis	Found dead on seventh day; apparently from toxemia, injection slough; liver repairing well
20	20-65	4	0.22	Iodozobenzoic acid 0.5 gm.	60% (+) necrosis	Operation on second day; found dead on fourth day; debris in liver largely removed; dog developed distemper during experiment
21	20-17	4	0.15	Iodoxybenzoic acid 1 gm.	10-15% necrosis; slightest possible trace fat	Recovered
22	20-57	3	0.21	Iodozobenzoic acid 0.8 gm.	No necrosis; no fat	Liver sections from operation show glycogen; died on seventeenth day from pneumonia; injection slough
23	20-76	3	0.21	Iodozobenzoic acid 0.8 gm.	45% necrosis; very severely injured up to ½	Recovered; very slight injury from injection
24	20-31	4	0.18	Iodoxybenzoic acid 0.5 gm.	Slightest possible trace necrosis; trace fat over ⅖	Recovered; injection slough
25	20-30	4	0.14	Iodoxybenzoic acid 0.5 gm.	45% necrosis	Recovered; injection slough

acid was dissolved in tenth normal sodium hydroxid about 0.1 gm. to 1 c.c., and the volume increased by addition of 10 per cent. acacia solution; no doubt the ugly sloughs obtained in such instances were due to the alkalinity of the solution (unfortunately much stronger than necessary), not to the drugs used; controls where alkaline solutions alone were used showed the same sloughs. Iodozo-benzoic acid was given suspended in 10 per cent. acacia solution; these injections did not cause such severe reactions, but the drug seemed to be absorbed only partially. With the exception of the sloughs already mentioned there have been no ill effects noted from injections of either drugs. On one occasion a large dose of iodozo-benzoic acid suspension was given to a dog intraperitoneally; this caused an acute reaction and contributed largely to the dog's death within a few hours (chloroform was given also, subcutaneously). Intraperitoneal injections of from 10 to 20 mg. iodozobenzoic acid in rats sometimes seem very toxic, in other cases apparently cause no reaction.

It is interesting to note the very small doses of chloroform which may prove fatal when given subcutaneously to dogs after a preliminary fast. Experiments 10 and 11 indicate the narrow zone of safety between a dose producing no injury and that causing death.

Experiment 22 indicates superficially a very marked protection, but since the liver showed glycogen at operation, it may be a food protection, either because of an inadequate starvation period, or because of feeding by mistake on fasting days.

Table 3 merely amplifies experiments recorded in Table 2 and adds Experiments 26 and 27 to complete the record of Dog 20-30. Experiment 26 will be taken up later in discussing the use of cyanids. Experiment 27 is merely a repetition of 17, to act as double control. This double check was attempted on Dog 20-76 (Table 2), but the second trial with chloroform alone showed only about 50 per cent. necrosis, compared with the original 60 per cent.; this is the only time that two controls on the same animal have failed to check, and this is not a very wide fluctuation considering that estimations are merely relative.

It will be noted that Experiment 17 was intended as a control not only for Experiment 25 on the same animal, but for Experiment 16 on Dog 20-31; while Experiment 24 controls Experiment 16 on the same animal. A point worth noting is that the weights of these dogs in control experiments were less than in the others; in such cases the animals actually received less chloroform; but in the cases with greater weights the surplus fat may have been more than sufficient as a block to chloroform to compensate for the greater injection, ignoring any possible effect from organic oxidizing agents. However, no such

TABLE 3.—EFFECT OF IODOXY-BENZOATE

	Experiment Number	Date of Injection	Fasting Period, Days	Weight at Time of Injection, Kilos	Chloroform, C.c. per Kilo (subcutaneously)	Potassium Cyanid (subcutaneously), Mg.	Iodoxy-benzoic Acid (subcutaneously), Gm.	Amount of Liver Injury	Remarks
Dog 20-30 Small male terrier	17	11/10/19	4	5.80	0.14	..	...	60% necrosis; slightest possible trace fat	Questionable distemper during convalescence; wound healed slowly
	25	12/18/19	4	6.42	0.14	..	0.5	45% necrosis; slight trace fat	Iodoxy-benzoic acid dissolved in alkaline acacia solution; injection slough
	26	2/ 4/20	4	6.30	0.14	25	...	45-50% necrosis; slight trace fat	Potassium cyanid dissolved in 12 c.c. physiologic sodium chlorid solution
	27	3/22/20	4	6.02	0.14	..	...	60% necrosis; slight trace fat	Experiment performed as second control
Dog 20-31 Young female collie	16	11/10/19	4	6.60	0.18	..	0.5	Slightest possible necrosis; trace of fat over ⅖ of each lobule	Iodoxy-benzoic acid dissolved in alkaline acacia solution; questionable distemper during convalescence; injection slough
	24	12/18/19	4	5.68	0.18	..	...	55% necrosis; slightest possible trace fat	Alkaline acacia solution injected; found dead on second day; necrosis at point of injection; edema and congestion in lungs

explanation will hold in the case of Dog 20-76, for here the weights in control experiments were practically the same, and both slightly greater than the weights in experiments when other substances were injected, in which latter cases the injuries sustained were less.

The tables and protocols which follow present a few experiments in which cyanid were used in conjunction with chloroform.

Experiment 28.—*Control; Chloroform Anesthesia.* Dog 19-74, young black and white male terrier.

Dec. 7, 1918: Weight, 11.8 pounds. In good condition; fourth day of fasting; chloroform anesthesia for one and a quarter hours.

December 9: Weight, 11.1 pounds. Active. Piece of liver removed in afternoon; bled freely. Sections show necrosis involving one-half of each liver lobule, and a moderate degree of fatty degeneration in zone surrounding necrosis.

Chloroform Anesthesia; Cyanid Subcutaneously. Dog 19-74, a black and white male terrier.

Oct. 18. 1919: Weight, 15 pounds. In good condition; isolated before daily feeding; fasting begun. Since the control experiment (given above) was performed, this animal has been subjected to two experiments in which organic oxidizing agents were injected intravenously (Table 1), also a second control with result the same as in the first.

October 19 and 20: No food.

October 21: Weight, 13.9 pounds. Active. Chloroform anesthesia for one and a quarter hours. Gave 15 mg. potassium cyanid in 30 c.c. physiologic sodium chlorid solution subcutaneously in divided doses at intervals during anesthesia.

October 23: Weight, 13.3 pounds. Active. Piece of liver removed at 1:30 p. m.; many adhesions; considerable bleeding; some subcutaneous edema in area of injections (due to alkalinity of potassium cyanid). Sections show approximately 60 per cent. necrosis.

Experiment 15.—*Control; Chloroform Subcutaneously.* Dog 20-76, a young, black mongrel, female.

Jan. 24, 1920: Weight, 14.2 pounds. Active and in good condition. Isolated before daily feeding; fasting begun.

January 25: No food.

January 26: (Third day of fasting.) Weight, 12.9 pounds (5.85 kilos). At 5 p. m. gave 1.23 c.c. chloroform (0.21 c.c. per kilo) plus 2.5 c.c. liquid petrolatum subcutaneously; temporary ether anesthesia.

January 27: Weight, 12.4 pounds. Quite active.

January 28: Weight, 12.3 pounds. Dog in fair condition. Piece of liver removed at 3:30 p. m. Sections show 60 per cent. necrosis; fat plus.

Experiment 30.—*Chloroform Subcutaneously; Cyanid Subcutaneously.* Dog 20-76, a young, black mongrel female.

March 19: Weight, 14.8 pounds. In excellent condition. Isolated after daily feeding; fasting begun.

March 22: (Third day of fasting.) Weight, 12.9 pounds (5.85 kilos). Gave 1.23 c.c. chloroform (0.21 c.c. per kilo) plus 2.5 c.c. liquid petrolatum subcutaneously; followed immediately by 35 mg. potassium cyanid in 35 c.c. physiologic sodium chlorid solution subcutaneously; temporary ether anesthesia.

March 23: Active. Full diet.

March 24: Active. Piece of liver removed at 3:15 p. m. Sections show from 35 to 40 per cent. necrosis. Point of injection of potassium cyanid later showed a little local necrosis (due to alkalinity of potassium cyanid).

TABLE 4.—POTASSIUM CYANID AS INFLUENCING CHLOROFORM LIVER INJURY

Experiment Number	Dog Number	Weight, Kilos	Fasting Period, Days	Chloroform	Potassium Cyanid (subcutaneously), Mg.	Liver Injury	Control Liver Injury (Same Dog)	Organic Oxidizing Agent Injection (Same Dog)	Remarks
28	19-74	6.3	4	1¼ hours anesthesia	15	60% necrosis	50% necrosis (chloroform anesthesia, no drugs—2 controls with same result)	50% necrosis (chloroform anesthesia; 0.14 gm. iodoxy-benzoic acid intravenously) 25% necrosis (chloroform anesthesia; 0.25 gm. iodozo-benzoic acid intravenously with question of feeding during starvation period)	Greater injury caused by potassium cyanid
29	20-17	6.6	4	0.15 c.c. per kilo subcutaneously	20	35-40% necrosis	35% necrosis (very severe injury up to ½)	10-15% necrosis (1 gm. iodoxy-benzoic acid subcutaneously)	A shade more necrosis in potassium cyanid experiment, but not so much surrounding injury
26	20-30	6.3	4	0.14 c.c. per kilo subcutaneously	25	45-50% necrosis	60% necrosis (2 controls with same result)	45% necrosis (0.5 gm. iodoxy-benzoic acid subcutaneously)	Less necrosis in potassium cyanid experiment than in controls; about the same as with organic oxidizing agent injection
30	20-76	5.9	3	0.21 c.c. per kilo subcutaneously	35	35-40% necrosis	(1) 60% necrosis (2) 50% necrosis	45% necrosis (0.8 gm. iodozo-benzoic acid subcutaneously)	Less necrosis in potassium cyanid experiment than in control or when using iodozo-benzoic acid

Between Experiments 15 and 30 came Experiment 23 in which the dog was given iodozo-benzoic acid subcutaneously (Table 2). In May this dog was used for another control similar to Experiment 15, and showed approximately 50 per cent. necrosis. In a final experiment in June the dog was given 26 mg. sodium cyanid (molecular equivalent of the 35 mg. potassium cyanid in Experiment 30), but 12 mg. of this were injected intraperitoneally and death occurred from respiratory failure in about one hour.

Experiment 35.—*Chloroform Anesthesia; Sodium Cyanid Intravenously.* Dog 21-18, a brindle mongrel, male; right eye shows keratitis and staphyloma, suggesting previous distemper infection.

Sept. 14. 1920: Weight, 25.5 pounds. In good condition and active. Isolated after daily feeding; fasting begun.

September 15, 16. 17. No food.

September 18: Weight, 23.8 pounds. Active. Chloroform anesthesia for forty-five minutes (3 to 3:45 p. m.). During thirty-five minutes of anesthesia 10 mg. sodium cyanid in 100 c.c. physiologic sodium chlorid solution were given intravenously (0.01 per cent. solution = approximately 1/500 N). Considerable chloroform was administered, but some respiratory difficulty was encountered because of sodium cyanid. More than the 100 c.c. would have been given except for shallow breathing, tendency to apnea, and a rapid, weak pulse.

September 19: Weight, 23.3 pounds. A little dull. Casein feeding.

September 20: Weight, 23.5 pounds. Somewhat dull. Casein feeding. Operation at 3 p. m. Piece of liver removed; liver is grossly injured. Sections show about 35 per cent. necrosis and a trace of fat.

Experiment 36.—*Chloroform Anesthesia; Physiologic Sodium Chlorid Solution Intravenously.* Dog 21-18. A brindle, mongrel male.

Oct. 19, 1920. Weight, 30.7 pounds. Bright and active. Wounds of former operations healed. Isolated after daily feeding; fasting begun.

October 20, 21 and 22: No food.

October 23: Weight, 27.5 pounds. Active and in good condition. Chloroform anesthesia for forty-five minutes (3:40 to 4:25 p. m.). Gave 100 c.c. physiologic sodium chlorid solution intravenously during anesthesia.

October 24: Weight, 27 pounds. Quite active. Gelatin feeding.

October 25: Weight. 25.5 pounds. Active. No vomiting. Operation at 2:30 p. m. Piece of liver removed; liver appears injured in gross. Sections show approximately 55 per cent. necrosis and a moderate amount of fat.

The experiments in which potassium cyanid was given subcutaneously appear very irregular and contradictory. On account of the immediate toxicity, the drug was given cautiously in rather small dosage; however, this accounts in no way for the fact that some cases show even less injury than the control experiments on the same animals; it may be coincidence that the larger doses of potassium cyanid (25 and 35 mg.) seem to have been somewhat protective. It will be recalled that we found no effect on chloroform liver injury from small intravenous injections of potassium cyanid.

Since in one case a small area of necrosis appeared at the point of injection of potassium cyanid, and in other cases moderate tissue edema

TABLE 5.—SODIUM CYANID INTRAVENOUSLY AS INFLUENCING CHLOROFORM LIVER INJURY

Experiment Number	Dog Number	Weight, Pounds	Fasting Period, Days	Chloroform Anesthesia, Min.	Sodium Cyanid Intravenously, Mg.	Liver Injury	Remarks
31	20-108	17.5	4	45	..	60% necrosis	Control. Recovery; operation on second day following anesthesia
32	20-112	14.8	4	45	..	70% necrosis	Control. Found dead on second day following anesthesia
33	20-119	28.6	4	45	18	60% (+) necrosis; fat very extensive almost to lobule peripheries	Sodium cyanid in physiologic sodium chlorid solution, 1 mg. to 10 c.c.; intervals of accelerated breathing during anesthesia; periods of apnea the last few minutes; recovery; operation on second day following chloroform
34	20-119	27.4	4	45	..	75% (+) necrosis; fat moderate	Control (see Experiment 33). Found dead on second day following anesthesia
35	21-18	23.8	4	45	10	35% necrosis; trace of fat	Sodium cyanid in physiologic sodium chlorid solution, 1 mg. to 10 c.c.; tendency to apnea and rapid, weak pulse, especially toward the last; recovery; operation on second day
36	21-18	27.5	4	45	..	55% necrosis; fat moderate	Control (see Experiment 35). 100 c.c. physiologic sodium chlorid solution intravenously during anesthesia; recovery; operation on second day

was manifested, we must conclude that a considerable local reaction took place (due in part to alkalinity of the drug), and it seems likely that not only the tissue suffered but that a certain part of the potassium cyanid may have been destroyed locally.

Table 5 gives the results of sodium cyanid injections in two dogs; these same animals were later used as controls; two other control experiments on different animals are also included. These experiments indicate that the cyanid may have some protective value against chloroform injury. The attempt was made to give the anesthetic at the same rate in all cases, but on account of respiratory difficulties caused by the cyanid it is possible that the animals receiving such injections were given somewhat less chloroform. This is suggested as a possibility only, but if so might account for the lessened liver necrosis in such cases. For comparative purposes we consider that controls on the same animals are by far the most valuable.

DISCUSSION

Although these experiments were undertaken to throw some light on the rôle of oxidations in chloroform liver injury, the results are far from conclusive. There appears to have been a definite amount of protection in the cases when organic oxidizing agents were injected in large amounts subcutaneously. Should this have been because an increased supply of oxygen was furnished to a liver hampered by its lack, we should have expected cyanids to have acted in an opposite manner, as found by Loevenhart, Amberg and their associates in various conditions; as a matter of fact, relatively large doses of cyanids seem to have been themselves rather protective in certain instances.

Administration of cyanids intravenously during chloroform anesthesia has been difficult because of respiratory irregularities; this trouble has been much more marked than in control experiments (without the cyanid). Loevenhart has had less difficulty in giving a solution of sodium cyanid five times as concentrated as that used by us. There are two factors in our experiments which may account, in part, for this high immediate cyanid toxicity: (1) preceding fasting period, and (2) simultaneous chloroform anesthesia.

We are safe in saying that the experiments do not prove that liver necrosis following administration of chloroform has any connection with a disturbance of normal tissue oxidation.

At the present state of our knowledge it is hardly justifiable to assume that chloroform narcosis and chloroform liver necrosis are manifestations of the same pharmacologic property of the drug; this point is often left rather indefinite in the literature on the subject. It is relatively simple to obtain one without the other. Since our observations relate entirely to the phenomenon of liver injury, we do not extend our deductions to bear at all upon the existing theories pertaining to the anesthetic action of chloroform.

SUMMARY

Very small amounts of chloroform given subcutaneously are sufficient to cause an extensive liver injury in fasting animals.

The intravenous injection of small amounts of organic oxidizing agents (iodoxy- and iodozo-benzoic acids and their sodium salts) seems to have no effect on chloroform liver necrosis. Large amounts of these substances subcutaneously appear to afford the liver some protection against chloroform injury.

The administration of cyanids (potassium and sodium) does not effect chloroform liver injury in any constant manner; it seems that large doses may exert some protective action.

These experiments offer no evidence that chloroform liver injury is a result of disturbance in tissue oxidations.

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STUDIES OF LIVER FUNCTION.

BENZOATE ADMINISTRATION AND HIPPURIC ACID SYNTHESIS.

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Much work has been done in an effort to understand the complex function of the liver and considerable information of value has been obtained. It is fair to say, however, that the estimation of functional capacity of the liver cannot as yet be made with any convincing accuracy. We have reason to believe that the liver has a very large factor of safety—that it can tolerate an extensive injury and yet carry on its essential body functions. Any satisfactory functional test must include, therefore, some factor of strain or load which can measure the upper limits as well as the lower limits of liver function. It is certainly possible that some of the many functions of the liver may be seriously impaired while others, perhaps of more fundamental significance, are not seriously disturbed. This may apply to acute as well as chronic disease conditions.

Probably the liver function test which is of most value in physiological experiments is given by the use of phenoltetrachlorphthalein as described by Whipple, Peightal, and Clark (1). This drug is given intravenously and eliminated promptly in the bile. It can readily be recovered from the feces and accurately estimated. This functional test shows a great impairment after extensive liver injury by chloroform or phosphorus, but a serious objection is at once obvious if any abnormalities of bile secretion are present. With complete biliary obstruction obviously the test is of little value and with chronic inflammation of the bile passages, we find a marked lowering in output of the phenoltetrachlorphthalein—for example in long standing biliary fistulas. For clinical use, therefore, this test has very serious limitations. We cannot rely in clinical diagnosis on any liver test which postulates a normal bile flow in normal bile passages.

The ideal liver functional test would consist in the introduction intravenously of some non-toxic substance which would test by a synthetic demand the functional reserve of the liver (normal or abnormal). The product formed by this synthesis should be obtainable from the blood rather than from the urine, as renal abnormality would not confuse the issue. This ideal functional test is not at hand but it appears to be a possibility and all attempts along these lines are worth while and should give information of value. We submit these experiments, which were undertaken as a part of this experimental program to study liver function.

Recognizing the importance of the liver in many synthetic endogenous processes, we felt it would be desirable to extend the study of hippuric acid synthesis under experimental conditions. It has been claimed by some that hippuric acid synthesis is a function of the kidney, by others a part of liver activity—in a word, the question is open to debate. We feel that our experiments furnish new data which will aid in the final solution of this complex physiological equation.

A critical review of the literature touching the influence of benzoic acid and benzoates upon the general metabolism of the experimental animal reveals the fact that the action of this drug is still far from being completely understood. The earlier investigators and particularly Schmiedeburg sought to establish the synthesis of hippuric acid from benzoic acid as a function of kidney activity, using principally what was considered at that time (1876) a very reliable method of transfusion of excised organs. These experiments have stood unquestioned until the last few years, when considerable evidence was adduced to show that the kidney was not alone responsible for hippuric acid production. The work of Kingsbury and Bell (2) with nephrectomized dogs and dogs in which an experimental nephritis had been produced shows little change in the hippuric acid synthesis from the previously normal condition and that in the dog at least the kidney is not the site of the reaction. In rabbits the evidence still appears to be conflicting. On the other hand, the known rôle of the liver in certain processes of metabolism and the influence of benzoic acid on metabolism as shown by our experiments suggests that the liver might play a definite part in the conjugation of benzoic acid with glycocoll. An analysis of the parenchyma of various organs, after the injection of benzoic acid, shows that there is a higher content of hippuric acid in the liver than in other organs (Kingsbury and Bell). Further evidence that the liver may be involved in the production of hippuric acid is offered by Lackner, Levinson, and Morse (3) who show a definite decrease of the hippuric acid elimination after a liver necrosis, which had been produced by hydrazine sulfate. Dogs were used and benzoate (0.5 to 2.0 gm.) was given by mouth.

The actual mechanism whereby the benzoic acid radicle is neutralized in the body is not understood. The fact that the amount of glycocoll eliminated as hippuric acid is much larger than the amount of glycocoll in the food has led to various explanations which need only to be mentioned in passing. Parker and Lusk (4) suggested the possibility of a glycocoll reserve in the body which can be "washed out" by successive doses of benzoate. The massive breaking down of body proteins is advocated by Ringer (5) and others. The synthesis of glycocoll from simple substances and simple amino-acids may occur (Epstein and Bookman, 6). Lewis maintains that products of metabolism, which might otherwise go to the formation of urea, may be diverted to the production of glycocoll (7) and this hypothesis is confirmed by the work of McCollum and Hoagland (8) who find a decrease in the urea nitrogen elimination of pigs on a carbohydrate diet following the administration of benzoates. Lastly the hypothesis of Umber (9), objected to by Abderhalden (10), that benzoic acid abstracts glycocoll from the globulin molecule of the tissues, thereby converting it to an albumin molecule; and that the action of benzoic acid in the body is merely to change the normal albumin-globulin ratio.

That so many theories exist is evidence that the process is either not understood completely, or that no one explanation is sufficient. Since all hypotheses of internal metabolism are deduced from the study of the urinary nitrogen, and since the conflicting theories advanced are based on conflicting results of urinary analysis, an attempt will be made to show that *different results may be referable to differences in animals used and to differences in dosage.*

Methods.

For the following experiments female dogs were kept in standard metabolism cages and the urine collected at a fixed hour each day. The 24 hour specimen included the cage collection, cage washings, bladder urine, and bladder washings made up to a standard dilution. The dogs were fasted for 3 or 4 days previous to the experiment to allow the urinary nitrogen excretion to reach a constant base line and food was withheld during the entire experiment. Water was supplied in the cage at all times and it was noted that after the injection of sodium benzoate the dogs showed a much greater thirst. The collected urine was subjected to the following analysis: Total urinary nitrogen by the Kjeldahl method; urea and ammonia nitrogen by Marshall's method; free benzoic acid by the method of Raiziss and Dubin (11); hippuric acid by the method of Folin and Flanders (12); all determinations were made in duplicate with suitable controls. A slight modification was made in both the method for the determination of the free benzoic acid and in the method for the determination of the hippuric acid.

Determination of Free Benzoic Acid. 100 cc. of urine are acidified by the addition of 1 cc. of strong nitric acid and 50 gm. of ammonium sulfate are added. To this are added, in a 250 cc. Erlenmeyer flask, 50 cc. of freshly distilled chloroform; the whole is then shaken thoroughly for some time and allowed to stand from 3 to 24 hours. At the end of this time, the chloroform is separated and is transferred into a second Erlenmeyer flask. The residue is washed into the first flask with 25 cc. of fresh chloroform and treated in the same way as the first extraction. The extract is washed by shaking with 100 cc. of saturated common salt solution and allowed to stand over night. On the next day the chloroform is separated in a clean dry funnel and is titrated with a standardized tenth normal alcoholic solution of sodium ethylate (phenolphthalein indicator). It was found convenient to keep the various chloroform fractions separate for the reason that, as was often found, the urine may be free from benzoic acid, a fact which can be noted on the first titration and further extraction with the chloroform rendered unnecessary. In each set of benzoic acid determinations (two daily for each dog) a complete duplicate was carried out with distilled water to insure no acid from the first acidification of the urine being retained in the extraction, and to show that the washing process had been sufficient.

Determination of Hippuric Acid.—100 cc. of urine are rendered alkaline with 10 cc. of 5 per cent sodium hydroxide and are evaporated to dryness on a water bath. The residue is dissolved in 25 cc. of water, and is then decanted into a Kjeldahl flask, and 25 cc. of strong nitric acid are poured into the same flask. A few crystals of copper sulfate are then added and the Kjeldahl flask is fitted with a long reflux condenser; an apparatus may be set up so that four or five determinations may be made at the same time with an extra flask for a control. The material in the flask is now boiled over a small flame for several (3 or 4) hours until the solution is a clear blue. When this stage is reached the condenser is allowed to cool and the inside is washed down twice with 25 cc. of distilled water. To the material in the flask, the volume of which is now about 100 cc., 50 cc. of freshly distilled chloroform are added and the whole is vigorously agitated for several minutes and is then allowed to stand for several hours. The chloroform is separated in the same way as in the method for the estimation of free benzoic acid and is thus similarly washed and titrated. As in the former method, it is advantageous to keep the extracts separate and to continue with extracting repeatedly until all the benzoic acid has been removed. It is hardly necessary to outline the rationale of the procedure at this time—reference has been made to the sources in which these methods were originally described, where additional and furthor information will be found.

Raiziss and Dubin (11) advise the use of toluene for the benzoic acid extraction, but in this work chloroform was found more convenient and it was determined experimentally that under the same conditions both solvents extract approximately the same amount of acid in the same time.

It will be seen that the extraction is made from a solution which is very strongly acid, and that if for some reason the chloroform is impure some of the nitric acid will be retained in the chloroform and the titration with the sodium ethylate will be rendered extremely inaccurate. It was found that small quantities of alcohol in the chloroform caused this error, and on this account it was necessary to wash the chloroform repeatedly with salt solution. When it was desired to reutilize chloroform which had been used in a previous extraction, it was rendered strongly alkaline and washed repeatedly. Since the used chloroform contained phenolphthalein, the washing in an alkaline solution was continued until the pink color had disappeared, and the chloroform was then considered ready for the first distillation. The distillation was carried out in the usual way, in a water bath, and the distillate washed by allowing it to stand for 24 hours over saturated salt solution, after which it was distilled a second time. Even the purest commercially obtainable chloroform was treated in this way, and it was found that such treatment was essential to avoid large and unexpected errors.

With the methods outlined above, it was possible in control experiments to recover from an aqueous solution of sodium benzoate an average of 99.8 per cent with a fluctuation of 10 per cent between extremes. The hippuric acid shows a recovery of 92.2 per cent with a fluctuation of 7 per cent between extremes.

It was found, in control experiments, that when bile, blood, or fecal material was added to the urine, and the urine was treated in the usual way for the determination of hippuric acid, that substances of an acid nature appeared in the chloroform, which were capable of neutralizing the sodium ethylate in the titration. The chemical nature of these substances was not satisfactorily determined. If the urine collected under the cage was found to be contaminated by blood or bile or if the fecal contamination was in the form of a diarrhea which could not readily be filtered out, the urine sample of that day was discarded for obvious reasons.

TABLE 1.

The Recovery of Hippuric Acid Before and After Anesthesia.

Dog 20–81. Brown spaniel, mongrel, adult.

Date.	Benzoate intravenously.		Hippuric acid.			Urea and ammonia N.	Total N.	Weight.
	Total.	Per pound.						
Jan. 24	Dog isolated and fasting begun.							
1921	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*lbs.*
Jan. 26	0	0	0.470	0.485	—	1.970	2.408	24.5
“ 27	0	0	0.425	0.440	—	2.040	2.632	24.0
“ 28	0	0	0.510	0.510	—	1.860	2.520	23.5
“ 29	0	0	0.542	0.510	—	1.880	2.380	23.3
“ 30	4	0.174	4.170	4.070	82.5	2.550	3.690	23.0
“ 31	4	0.176	4.850	5.140	102.0	2.340	3.660	22.8
Feb. 1	4	0.178	—	—	—	2.460	3.860	22.5
“ 2	0	0	—	—	—	2.460	3.470	21.8
Feb. 2	Chloroform anesthesia—60 minutes.							
Feb. 3	4	0.187	—	—	—	—	5.040	21.5
“ 4	4	0.190	5.140	4.400	93.2	—	5.150	21.0
“ 5	4	0.200	4.985	4.700	98.6	3.030	4.200	20.0

Except where specifically stated to the contrary, the benzoate was administered in the form of sodium benzoate, and injected into the jugular vein of the dog in a 5 per cent aqueous solution at the rate of about 20 cc. per minute. In the experiments marked "by mouth" the drug was given through a stomach tube, which was washed out with water.

To determine the effect of chloroform anesthesia upon the synthesis of hippuric acid several experiments were conducted, of which Tables I to III inclusive may be taken as typical examples. It has been shown by Davis and Whipple (13) that after 3 days fasting, 60 minutes of light chloroform anesthesia will produce a central necrosis involving one-half of the liver parenchyma in most cases, usually followed by recovery and liver regeneration. We must refer to the paper of Davis and Whipple (13) for the data establishing the constancy of liver injury under uniform conditions.

TABLE II.

The Recovery of Hippuric Acid in the First 5 Hours after Benzoate Injection Intravenously.

Dog 20-25. Fox terrier, adult.

Date.	Benzoate dose.	Time.	Hippuric acid.			Weight.
1920	*gm.*	*hrs.*	*gm.*	*gm*	*per cent*	*lbs.*
Oct. 30	3	1–5	1.940	1.750	55.4	22.0
Nov. 3	3	1–5	1.520	1.700	48.0	21.0
" 4	3	1–5	1.820	1.860	55.5	21.5
" 5	3	1–5	1.895	2.070	59.7	21.5
" 17	3	1–5	1.615	1.700	50.0	21.0
" 24	3	1–5	2.340	2.080	66.5	22.0
" 25	3	1–5	2.380	2.520	74.0	22.0
Dec. 1	Chloroform anesthesia—50 minutes.					
Dec. 2	3	1–5	1.265	1.265	38.2	20.5
" 4	3	1–5	1.225	1.270	37.6	19.5
" 6	3	1–5	0.925	0.945	27.9	19.0
Dec. 21	Dog dead from distemper—recovery from chloroform poisoning.					

Diet of bread and milk throughout experiment except on the 2 days preceding chloroform anesthesia.

From the examination of the percentage amounts of hippuric acid recovered (Table I), it will be seen that the recovery on 2 successive days average 92.2 per cent. After 60 minutes of chloroform anesthesia, the average recovery on 2 successive days is 95.5 per cent per 24 hours. The total recovery, therefore, is

TABLE II-A.

The Recovery of Hippuric Acid in the First 5 Hours after Benzoate Injection. Fatal Liver Injury.

Dog 20-21. Airedale, adult.

Date.	Benzoate dose.	Time.	Hippuric acid.			Weight.
1920	*gm.*	*hrs.*	*gm.*	*gm.*	*per cent*	*lbs.*
Nov. 6	3	1–5	2.150	2.00	63.1	21.5
Nov. 24	Chloroform anesthesia—60 minutes.					
Nov. 25	3	1–5	1.100	—	33.1	21.5
Nov. 25	Dog died, acute chloroform poisoning—extreme liver necrosis.					

not influenced by severe liver injury. From this and other experiments of a similar nature, it may be concluded that extensive liver necrosis due to chloroform has very little effect upon the total amount of hippuric acid synthesized during the whole 24 hours.

The possibility suggested itself that there might be a delay in this synthetic reaction. With this in mind, the 24 hour period

TABLE III.

The Recovery of Hippuric Acid from the 5th to the 24th Hour after Benzoate Injection Intravenously.

Dog 20–17. Small black mongrel, adult.

Date.	Benzoate dose.	Time.	Hippuric acid.			Weight
1920	*gm.*	*hrs.*	*gm.*	*gm.*	*per cent*	*lbs.*
Dec. 23	3	5–24	2.025	2.180	63.70	21.8
" 24	3	5–24	2.010	—	60.60	22.5
" 25	3	5–24	1.980	1.690	55.25	22.0
Dec. 29	Chloroform anesthesia—60 minutes.					
Dec. 31	3	5–24	2.400	(1.850)	64.05	21.0
1921						
Jan. 1	3	5–24	2.480	2.380	73.35	20.0
" 2	3	5–24	2.010	—	60.60	20.0
" 3	3	5–24	2.080	2.090	62.80	19.0

Complete recovery.

was arbitrarily split so that the first collection was made 5 hours after the injection of benzoate and the second collection comprised the specimen of the subsequent 19 hours.

From an examination of Table II, it will be seen that the amount of hippuric acid synthesized and excreted during the first 5 hours after the injection of sodium benzoate averages for a period of 7 days 58.4 per cent of the amount injected; while after 50 minutes of chloroform anesthesia the average for a 3 day period drops to 34.5 per cent. In this experiment, after a period of 2 days fast, chloroform anesthesia was given for 50 minutes. Except for this interval the dog was fed bread and milk. The dog died 20 days later from distemper after recovery from the chloroform poisoning.

Similarly in Table II–A, the recovery during the first 5 hours after the injection of sodium benzoate in the control period averaged 63.1 per cent, while after 60 minutes of chloroform anesthesia it averaged 33.1 per cent. In this experiment, although the bread and milk on which this dog was fed was withheld for only 2 days previous to the chloroform anesthesia, an extreme liver necrosis was produced and the dog died the following day. Histological examination of liver sections shows practically a complete liver cell destruction with only a few small groups of liver cells surviving about the portal tissues.

A liver necrosis was produced in a dog (Table III) by allowing the dog to fast for 2 days and then giving 60 minutes of chloroform anesthesia. Although the lesions thus produced may have comprised one-half or more of the liver parenchyma, the dog recovered from the effect of the intoxication. The effect on the hippuric acid output during the interval between the 5th and the 24th hour is much less marked than is seen in Table II. Before the period of anesthesia in an average of 3 days' determinations, the dog was excreting 59.8 per cent of the dose between the 5th and the 24th hour. After the period of anesthesia, the dog excreted 65.2 per cent during the same time. The dog was fed bread and milk except on the 2 days preceding the chloroform anesthesia.

On the basis of the tabulated and other experiments it would seem proper to conclude that a liver necrosis involving more than one-half of the liver parenchyma produces a *definite delay in the* *hippuric acid conjugation and excretion,* so that during the first part of the 24 hours the excretion is relatively less than during the later part of the 24 hours. The total amount of hippuric acid synthesized seems to be little altered if the whole 24 hour period is considered. It will be a matter of dispute whether on the basis of these experiments a further deduction can be made that the synthesis of hippuric acid is a function of the liver. The absence of demonstrable lesions or pathological conditions elsewhere in the body following chloroform anesthesia make it seem probable that the lesions in the liver are the cause of these changes noted in the excretion of the hippuric acid. Control experiments show that dogs injured by chloroform do not present an impairment of renal function to explain the delay in excretion of hippuric acid.

In these experiments it will be seen that the amount of hippuric acid recovered approaches 100 per cent of the amount injected. This, however, was not a constant finding. Dogs of the same weight and under the same conditions, show considerable variation in the amount of hippuric acid eliminated. No correlation could be established between the size of the dog, the size of the dose, and the amount eliminated. In some cases the amount recovered was only 70 per cent of the dose administered, and yet no free benzoic acid was recovered from the urine. These abnormalities deserve further study.

The Rise in Urinary Nitrogen Following Sodium Benzoate Injection.

A number of experiments (nine) were carried out to observe the effect of sodium benzoate in varying doses on the urinary nitrogen, and as the results are very similar, only a few typical examples need be given. Table IV shows the typical rise in urinary nitrogen following the injection of 3.5 gm. of sodium benzoate in a 5 per cent solution into the jugular vein of a 15 pound dog. In this case the total urinary nitrogen excreted averages 1.596 before the injection of the benzoate, and rises to an average of 2.968 gm. after the injection. The greatest part of this rise (excluding the fraction contained in the hippuric acid) is made up of urea, which rises to an average of 2.008 gm. from a normal average of 1.005 gm. The ammonia shows a definite rise over the normal period.

TABLE IV.

The Rise in Ammonia, Urea, and Total Urinary Nitrogen Following Benzoate Intravenous Injection.

Dog 20-96. Small black mongrel, senile.

Date.	Benzoate intravenously.		Hippuric acid.			Urea N.	Ammonia N	Total N	Weight.
	Total.	Per pound.							
1920	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm*	*gm.*	*gm.*	*lbs.*
June 24	0	0	0.236	0.286	—	1.085	0.135	1.568	15.00
" 25	0	0	0.498	0.398	—	0.995	0.106	1.652	14.75
" 26	0	0	0.286	—	—	0.935	0.134	1.568	14.50
" 27	3.5	0.185	3.980	3.680	95.2	1.780	0.268	2.688	14.25
			4.070						
June 28	3.5	0.188	4.070	3.750	96.5	1.880	0.204	2.884	14.00
" 29	3.5	0.191	3.640	3.300	94.0	2.350	0.224	3.332	13.75
" 30	0	0	—	—	—	0.558	0.157	1.064	13.50

TABLE V.

The Effect of Gradually Increased Doses of Benzoate on the Urinary Nitrogen.

Dog 20-77. Small fox terrier, brown, adult.

Date.	Benzoate intravenously		Hippuric acid.			Urea N	Ammonia N.	Total N.	Weight.
	Total.	Per pound.							
1920	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*lbs.*
Apr. 3	0	0	0.045	—	—	0.739	0.282	1.663	11.00
" 4	0	0	0.110	—	—	0.700	0.590	1.624	10.75
" 5	0	0	0.066	—	—	0.850	0.269	1.512	10.50
" 6	0	0	0.117	—	—	0.810	0.380	1.456	10.00
" 7	0	0	0.088	—	—	0.950	0.095	1.512	10.00
" 8	1.25	0.128	1.380	1.305	94.6	1.120	0.176	1.680	9.75
" 9	1.50	0.158	1.230	1.116	70.0	1.410	0.213	2.016	9.50
" 10	2.00	0.222	1.845	1.775	80.6	1.170	0.240	2.167	9.00
" 11	0	0	0.279	—	—	0.950	0.112	1.512	8.50
" 19	0	0	0.235	—	—	0.660	0.045	1.036	9.50
" 20	0	0	0.252	—	—	0.760	0.067	1.148	9.50
" 21	0	0	0.244	—	—	0.747	0.101	1.232	9.25
" 22	0	0	0.219	—	—	0.803	0.112	1.204	9.25
" 23	2.50	0.278	2.100	1.800	67.5	1.422	0.224	2.142	9.25
" 24	3.00	0.334	2.360	2.130	64.2	1.544	0.246	2.240	9.00
" 25	4.00	0.445	Lethal dose.						

Table V shows the effect of gradually increasing the dose of the sodium benzoate. As the dose is increased from 1.25 to 4 gm., it will be seen that there is a progressive increase in the total urinary nitrogen. The increase in the urea nitrogen corresponds somewhat to the increase in total urinary nitrogen but the relation of the percentage of urea nitrogen to the total urinary nitrogen is very variable. In this experiment doses varying from 0.128 to 0.222 gm. per pound dog fail to produce a definite rise in ammonia, while larger doses seem definitely to increase the ammonia. During the first part of the experiment, the variation in the ammonia is within the physiological variation, but after the 19th, a lower normal base line is maintained which is definitely exceeded after the injection of larger doses. A lethal dose was reached with 0.445 gm. per pound body weight.

TABLE VI.

Benzoate Given by Mouth and Intravenously.

Dog 20-101. Airedale. pup, weight 20 pounds.

Date	Benzoate.		Hippuric acid.			Urea N.	Ammonia N.	Total N.	Remarks.
	Total.	Per pound							
192	*gm*	*gm*	*gm*	*gm.*	*per cent*	*gm.*	*gm*	*gm.*	
May 8	0	0	0.295	—	—	1.530	0.153	2.380	
" 9	0	0	0.388	—	—	1.395	0.140	2.296	
" 10	0	0	0.338	—	—	1.260	0.134	2.212	
" 11	0	0			—	1.345	0.112	2.128	
" 12	0	0	0.346	—	—	1.275	0.129	2.184	
" 13	3.0	0.113	3.320	2.610	79.0	1.715	0.258	2.800	Intravenous.
" 14	3.5	0.138	3.880	3.420	88.0	1.760	0.258	2.940	"
" 15	4.0	0.152	4.425	3.830	87.0	1.955	0.244	3.136	"
" 16	0	0	—	—	—	1.155	0.145	2.156	
" 17	0	0	—	—	—	1.382	0.168	2.100	
" 18	3.0	0.113	2.500	2.150	65.0	1.125	0.134	2.016	By mouth.
" 19	3.5	0.135	3.430	3.100	79.8	1.170	0.370	2.268	" "
" 20	4.0	0.154	4.025	3.750	85.5	1.585	0.291	2.660	" "
" 21	0	0	0.512	—	—	1.300	0.179	2.080	

To determine whether the method of administration of the drug exerts any influence on its metabolism through possible variations in rate of absorption, injections of the same amount of sodium benzoate were given intravenously and later by mouth to a dog under as nearly as possible identical conditions. The results of this experiment may be seen in Table VI.

In this experiment the usual marked rise in total urinary nitrogen, urea nitrogen, and ammonia nitrogen follow the intravenous injection. The first dose by mouth was partly vomited, which accounts for the low value of the recovered hippuric acid. On the 19th and 20th, the full dose was retained. It will be seen that the response to the ingestion is very much less marked than the response to the intravenous injection, the urea and total urinary nitrogen being almost within the limits of physiological variability and the ammonia alone showing a very slight rise.

TABLE VII.

Composite Table Showing the Total Urinary Nitrogen with Doses of Sodium Benzoate Varying from 0.079 to 0.445 Gm. per Pound.

Dog No.	Total urinary nitrogen, 3 days' average.	Total urinary nitrogen after injection of benzoate.			Dose of sodium benzoate given per pound.			Dog weight.
		1st day	2nd day	3rd day	1st day	2nd day	3rd day	
					gm.	*gm.*	*gm.*	*lbs.*
20–101	2.306	2,352	2.464	2.372	0.079	0.090	0.102	20.00
20–101	2.240	2.800	2 940	3.136	0.113	0.138	0.152	20.00
20–77	1.553	1.680	2.016	2.167	0.128	0.158	0.222	9.70
18–38	2.760	4.440	3.860	3.330	0.146	0.150	0.150	19.00
20–81	2.485	3.690	3.660	3.860	0.174	0.176	0.178	22.00
20–96	1.596	2.668	2.884	3.332	0.185	0.188	0.191	14.30
20–20	1.344	2.016	2.240	2.240	0.200	0.200	0.200	12.10
18–38	2.760	3.466	3.819	2.979	0.216	0.216	0.220	19.00
20–20	1.222	3.024	3 416	3.248	0.286	0.292	0.298	12.10
20–77	1.155	2.142	2.240	Lethal.	0.278	0.334	0.445	9.70

Table VII shows that below a dosage of 0.140 gm. of sodium benzoate per pound of body weight, the change in the total urinary nitrogen is not significant. Doses larger than this, however, produce a definite and constant rise in urea, ammonia, and total urinary nitrogen. The greatest responses noted were between the doses of 0.180 and 0.200 gm. per pound. In three dogs with increasing dosage the rise in total urinary nitrogen was progressive to a certain point after which there was a less marked rise; this is shown in Dogs 20–81 and 18–38, and to a lesser degree in Dog

20–20. A sufficient number of observations are not available to determine whether this is a constant finding.

The fasting dog presents a constant base line of nitrogen excretion and the sudden rise following the injection of sodium benzoate must clearly be due to the breaking down of body proteins. This breaking down of body proteins was prevented by Epstein and Bookman (6) by the stimulating administration of dextrin, an observation we were able to confirm, as indicated in Table VIII.

TABLE VIII

The Rise of Urinary Nitrogen Following Benzoate Injection Prevented by Administration of Dextrin.

Dog 20–20. Fox terrier, adult.

Date.	Dextrin.	Benzoate.		Hippuric acid			Urea N.	Ammonia N.	Total N.	Weight.
		Total.	Per pound							
1920	*gm.*	*gm*	*gm*	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*lbs.*
May 8	0	0	0	0.210	—	—	0.715	0.075	1.288	13.00
" 9	0	0	0	0.236	—	—	0.890	0.067	1.232	13.00
" 10	0	0	0	0.270	—	—	1.240	0.112	1.624	12.75
" 11	0	0	0	0.442	—	—	0.990	0.177	1.512	12.50
" 12	0	0	0	0.362	—	—	0.920	0.190	1.456	12.50
May 13	0	3.5	0.286	3.510	3.180	88.5	2.260	0.277	3.024	12.25
" 14	0	3.5	0.292	3.720	3.390	96.0	2.390	0.274	3.416	12.00
" 15	0	3.5	0.298	3.540	3.210	90.5	2.240	0.230	3.248	11.75
May 16	0	0	0	0.440	—	—	1.540	0.151	2.172	11.25
June 3	0	0	0	0.218	—	—	0.985	0.089	1.148	12.25
" 4	0	0	0	0.236	—	—	0.975	0.106	1.148	12.00
" 5	0	0	0	0.210	—	—	0.965	0.095	1.262	11.75
" 6	0	0	0	0.270	—	—	0.965	0.078	1.204	11.50
" 7	0	0	0	0.285	—	—	1.020	0.095	1.260	12.00
June 8	30	0	0	0.220	—	—	0.780	0.095	1.064	12.00
" 9	30	0	0	0.255	—	—	0.492	0.218	1.148	12.00
" 10	30	0	0	0.264	—	—	0.589	0.078	0.800	11.75
June 11	30	3.5	0.306	3.440	3.350	86.5	0.962	0.185	1.344	11.50
" 12	30	3.5	0.306	3.640	3.390	87.5	0.995	0.525	1.624	11.50
" 13	30	3.5	0.313	4.040	3.770	97.0	0.975	0.224	1.652	11.25

A dog fasted for 7 days, during the last 4 of which his normal ammonia, urea, and total urinary nitrogen were determined. A dose of 3.5 gm. of sodium benzoate was injected which practically doubled the urea and total urinary nitrogen. After a period of rest, the same dog again fasted 7 days, during the last 4 of which the same determinations were made and a new base line established. 30 gm. of dextrin were given by mouth, which caused a slight drop in the nitrogen excretion. This was accompanied after 3 days by the same dose of benzoate which had previously caused such a marked rise in nitrogen elimination and little change in the nitrogen output occurred, showing that the dextrin had succeeded in preventing the reaction observed in the control period.

The Effect of Sodium Benzoate Injections on the Blood Serum Proteins.

Several experiments were carried out to determine the effect of benzoate injections on the blood proteins.

Dog 20–101 is a characteristic example.

	Before injection.	After injection.
	per cent	*per cent*
Non-proteins	2.15	2.15
Albumin	3.53	3.71
Globulin	1.69	1.42
Total protein	5.22	5.13

In this experiment, 4 gm. of benzoate were injected and the second determination was made 2 hours after the injection into a 20 pound dog. Determinations were made by the refractometric method of Robertson (14).

If we assume the blood plasma volume of the dog to be 5 per cent of his body weight, the serum globulin 1.7 per cent of his blood plasma volume, and the glycocoll 3.5 per cent of the serum globulin, the dog should have 0.270 gm. of glycocoll combined in the form of serum globulins. On the other hand, 4 gm. of sodium benzoate combine with 1.85 gm. of glycocoll to form hippuric acid, which is actually seven times the amount present in all the blood serum globulins. It is interesting to note that the globulin-albumin ratio remains the same while this demand for glycocoll is being met. The injection of benzoate does not influence the non-protein nitrogen circulating in the blood.

DISCUSSION.

Our experiments show that a very severe and even fatal liver necrosis due to chloroform may not diminish the total 24 hour amount of synthesized hippuric acid following administration of a unit dose of sodium benzoate. This may suggest that the liver has nothing to do with hippuric acid synthesis, but we believe this conclusion is not justified. Further study shows (Table II) that under conditions of liver injury, there is a *delay in the synthesis and excretion of hippuric acid* in the 5 hour period following the administration of the sodium benzoate. There is no decrease in renal function during this period as indicated by phthalein functional tests. The liver inquiry, therefore, *delays* this reaction which is so prompt in the normal dog. Our belief is that the liver is much concerned in the normal synthesis of hippuric acid in the body but after injury this function may be taken over in large measure by other cell protoplasm. This indicates too that other organs and tissues may take part in this synthetic reaction even under normal conditions.

Our experiments show a definite rise in ammonia, urea, and total urinary nitrogen following the *injection* of sodium benzoate into the circulation of the dog, whenever a certain dosage is exceeded. If these conditions be applicable to other animals, it would seem possible that the failure of some observers to note a rise in urinary nitrogen after administration of benzoate may be explained by the use of small doses. The rise in urea nitrogen is not in agreement with the observations of Ringer, and McCollum and Hoagland, who found that large doses of benzoate decrease the urea nitrogen excreted. It must be remembered, however, that McCollum and Hoagland worked with pigs, and that the caloric requirements of the animals were supplied, whereas in our experiments, the dogs were fasting. In both instances, however, the nitrogen of the urine was wholly endogenous. Our doses per unit body weight exceed those of McCollum and Hoagland and this may explain the differences.

How may we explain this rise in urinary nitrogen following the intravenous injection of sodium benzoate? Perhaps a tentative explanation may be advanced as follows: When the drug is given intravenously, the demand for conjugation is very acute and lacking the available glycocoll, there is a breakdown of body protein to supply a part of this emergency requirement. We may imagine that certain elements (glycocoll) are removed from the large protein molecule which as a result disintegrates, at least in part. The less acute demand made by administration of the drug by mouth can be met by the usual body mechanism (glycocoll synthesis) unless very large doses are given and there is no emergency breakdown of body protein. The death of the animal we may attribute to this destructive action which may break down body protein in such fashion that certain poisonous split products are formed in sufficient amount to cause fatal intoxication.

The interesting suggestion that glycocoll may be obtained from the globulins of the body finds no support in our experiments. It is known that under certain conditions there may be a rather prompt shift in the albumin-globulin ratio in the blood plasma. Therefore, this suggestion that glycocoll might be furnished in this way by a change of globulin to albumin was worthy of serious consideration. It may be claimed that our experiments do not rule out this possibility but at least we can say that the plasma albumin-globulin ratio does not change after a benzoate injection. If we wish to cling to this hypothesis, we must postulate an effect which is limited to the *tissue globulin-albumin ratio*—this would seem to be indeed a venture into realms of conjecture.

SUMMARY.

The synthesis of hippuric acid in the body following benzoate administration is not prevented by an extensive chloroform liver necrosis. A severe liver injury, however, will cause a distinct *delay* in the synthesis and excretion of hippuric acid. This indicates that the liver normally takes part in this synthesis but that other cell protoplasm of the body may be concerned in this conjugation and may in an emergency take over a greater part of the hippuric acid synthesis. This may apply particularly to the intravenous administration of the benzoate.

Our experiments show distinct increases in ammonia, urea, and total urinary nitrogen wherever dosages of benzoate are given intravenously, exceeding a certain amount per pound body

weight. The question of dosage may explain many discrepancies noted in the literature.

Under certain conditions benzoate injection causes a considerable protein breakdown due probably to the acute need for glycocoll which is taken from the body protein molecule. The suggestion that under emergency conditions the glycocoll may be obtained from the globulins finds no support in our experiments. The serum albumin-globulin ratio is not changed by administration of large doses of benzoate.

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BLOOD VOLUME STUDIES

V. The Carbon Monoxide Method—Its Accuracy and Limitations

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Many investigators consider the whole subject of blood volume to be in a state of hopeless confusion. Say our critics, how can we think otherwise when one set of observers report, the blood volume of dogs as 10 per cent body weight (dye methods), another as 8 per cent (CO method) and still others as 7 per cent (Welcker method). In this series of papers we submit experimental data which go far to explain these discrepancies and point the way to a blood volume method which is reasonably accurate (5 per cent error). In a word, our experimental data indicate that the blood plasma volume is accurately determined as 5 per cent (5 cc. per 100 gram body weight) by a number of dye methods. Furthermore our data show that the total body hemoglobin is accurately determined as approximately 4 per cent by the CO and Welcker methods. But in our opinion the true *total blood volume* is 5+ 4 = 9 per cent body weight, and not 10 per cent or 8 per cent respectively, calculating the hematocrit as 50 per cent. The *fundamental error, therefore, is the assumption that the ratio of cells to plasma is a constant* in all parts of the circulation. This we believe is contrary to fact. Experimental data and discussion of related facts are submitted below in their proper place (paper VII of this series).

It is not necessary to review the different methods and blood volume values as we can refer to a recent discussion by Salvesen (4). We may note in passing that the dilution blood volume methods of Cohnstein and Zuntz (1) which depend upon injection of isotonic sodium chloride solutions have no further usefulness. The recent experiments of Smith and Mendel (5) demonstrate the rapidity with which isotonic salt solutions leave the blood stream. Much of this solution may disappear within 5 minutes. Some observations in this laboratory on dogs confirm this work of Smith and Mendel on rabbits.

It has been assumed by some workers that the wide divergence in blood volume values might be explained by differences in technical procedure. We thought it highly desirable that these various methods be used in this laboratory with a uniform technique, using the same healthy animals under controlled conditions. For this reason we have described in much detail the various technical procedures and subjected our methods to a variety of control tests.

The carbon monoxide method is very simple in principle. The CO is inhaled and a sample of blood is withdrawn for analysis to determine the amount of contained CO. This gives the amount of dilution effected by the circulating blood and so the total circulating hemoglobin or red cells. It is assumed that the CO is held only by the hemoglobin of the red blood cells, but there is good reason to believe that the hemoglobin of the red marrow and striated muscles (myohematin) participate in this dilution (see paper VII). The carbon monoxide method was first used by Gréhant and Quinquaud (2), later modified by many others and recently by Van Slyke and Salvesen (4), who report a simple method for the accurate analysis of small amounts of CO in blood. This last method with a few minor modifications has been used in our work.

TABLE 1

Known amounts of CO mixed with deoxidized blood and quantitatively recovered

EXPERIMENT	CO MIXED WITH BLOOD	READING AFTER PYROGALLIC ACID	READING AFTER COPPER SOLUTION	CO RECOVERED FROM BLOOD
	cc.	*cc.*	*cc.*	*cc.*
I	0.265	0.285	0.02	0.265
II	0.265	0.275	0.02	0.255
III	0.390	0.400	0.02	0.380
IV	0.310	0.330	0.02	0.310
V	0.330	0.345	0.02	0.325
Average......	0.312			0.307

Method of administering the carbon monoxide. The apparatus used for giving the CO gas corresponds in principle with that arrangement employed by Gréhant and Quinquaud (2), later modified by others, as reviewed in the introduction. The technique in detail is as follows: The gas, made by mixing and gently heating formic and sulphuric acids in tube *A*, figure 1, is first burned at *B* until it gives a bright blue flame. The three-way stopcock of the tube is then turned so that the gas bubbles through the bottles *C*, which contain concentrated NaOH to remove any sulphur dioxide, carbon dioxide and water present. The carbon monoxide runs into the 100 cc. graduated burette *D*, previously filled with water and supplied with a leveling bulb, *D'*. This is connected by one arm of a three-way stopcock with a 50 cc. burette *E*, also previously filled with water and supplied with a leveling bulb, *E'*. The gas is run through these burettes and out through the arm of another three-way stopcock to *B* again, where about 200 cc. are burned, thus thoroughly rinsing out the connections. The two burettes are then filled with gas and all stopcocks closed except the one at *A* which is turned so as to allow the CO still generating to escape to the outside. The connecting tubes are clamped at *X* and *Y*. The gas in *E* is measured at atmospheric pressure by means of the leveling bulb and 50 cc. are run up into the graduated cylinder *F*, which contains a solution of copper sulphate in concentrated hydrochloric acid (prepared by Sandmeyer's method). The solution on gentle shaking for a few seconds absorbs all the CO leaving behind the impurities, the volume of which is read directly at atmospheric pressure with the leveling bulb, *F'*. The gas is then run out of *F* by raising the leveling bulb and opening the stop-cock. The solution is thus ready for the next analysis.

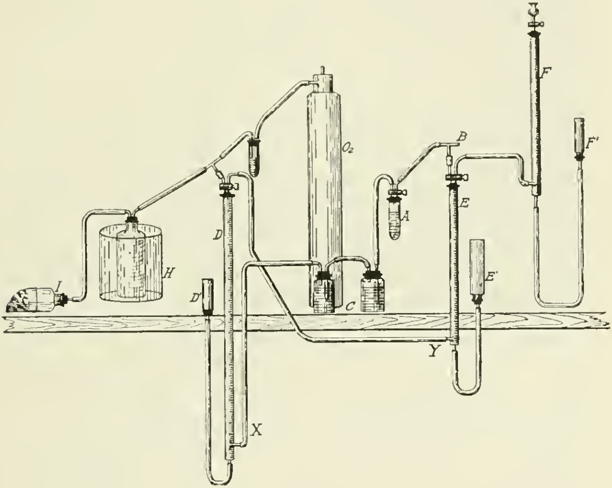

Fig. 1

The respiratory chamber, *H*, consists of a tank half filled with concentrated NaOH in which stands a bell-jar of about 2000 cc. capacity. The available gas space is 1000 cc. This is connected with *D* through the other arm of the three-way stopcock and also with the oxygen tank, as shown in figure 1. It is also connected with the nosepiece, *I*. This consists of a glass bottle from which the bottom has been removed, of size varying according to the weight of the dog. A piece of automobile inner tubing of suitable diameter and length is slipped over the bottom of the bottle so as to form a cap for the dog's nose and protect the animal from the glass edge.

To prepare the respiratory chamber the air is sucked up to a mark, leaving about 500 cc. of air in the chamber and the tube to the hood is clamped. Oxygen is then allowed to enter in sufficient quantity to keep a slightly negative pressure within the respiratory chamber. This prevents any outflow of CO during the exhalation. The O_2 makes a smaller respiratory chamber possible and prevents any discomfort to the dog due to deficiency of oxygen. The apparatus is now ready for use.

The dog is tied on its back with the head at the edge of the table. The neck over the jugular vein is shaved and the nose is well greased with crude vaseline, as is also the inside of the nosepiece. This is slipped over the dog's nose to completely cover the mouth and nostrils and held firmly during the inhalation. The tube to the respiratory chamber is immediately unclamped. When all is adjusted, a measured quantity of CO (sufficient to saturate the blood corpuscles one-third if one assume 100 cc. of blood per kilogram body weight, of O_2 capacity 21 cc. per 100 cc. blood) is run in, and the tube rinsed with oxygen. The dog breathes into the respiratory chamber for 6 minutes. During this time as the volume of the gas in the chamber decreases, due to absorption of carbon dioxide, oxygen is supplied from the tank to keep the quantity of gas practically constant.

At the end of 6 minutes the nosepiece is removed. Four minutes later two 5 cc. samples of blood are drawn, by means of a hypodermic syringe, from the jugular vein and placed in tubes with powdered sodium oxalate to prevent clotting. A 10 cc. sample is then taken in a hematocrit tube containing 2 cc. of 1.6 per cent sodium oxalate (isotonic for blood). A sample of air from the respiratory chamber is taken and analyzed as described later.

The purpose of this method is to introduce into the animal's blood stream a known amount of CO. It is therefore essential that there should be no dead space and that the residue of CO left in the respiratory chamber and dog's lungs be accurately determined. The possible dead space is the tube from *D* to *H*. This is eliminated by the inhalations of the dog combined with the flooding of the tube with oxygen. The method employed in analyzing the respiratory chamber air will be discussed shortly. The entire procedure from making the gas to taking the samples is readily carried out and may be completed within 30 minutes.

Method of analyzing the samples of blood. The samples of blood are analyzed in the Van Slyke apparatus for blood gas analysis (6). The method employed is that described by Van Slyke and Salvesen (7). Our technique in detail is as follows: Three cubic centimeters of 0.4 per cent ammonia are introduced into the 50 cc. chamber of a carefully calibrated Van Slyke apparatus. This is followed respectively by 3 drops of caprylic alcohol and 3 drops of a 1 per cent aqueous solution of saponin. Following this 3 cc. more of the ammonia solution are added. The chamber is evacuated and shaken a few times to extract the air. This is expelled and the process repeated to make certain that all the air is removed and that there is no leak around the stopcocks. On opening the lower stopcock, the solution should rise with a click to the top. Two cubic centimeters of this solution are run up into the cup. A 2 cc. Ostwald pipette, calibrated for complete delivery, is used to introduce the sample of blood into the cup under the ammonia solution. The blood and supernatant liquid are run into the chamber and the cup rinsed with a few drops of ammonia solution. One minute is allowed for laking. One cubic centimeter of a saturated potassium ferricyanide solution is then added, the stopcock sealed with mercury, and the apparatus evacuated until only about 2 cc. of mercury remain in the 50 cc. chamber. The machine is shaken for 15 minutes by a motor which throws the solution gently with a rotary motion against the sides of the chamber, but does not shake so violently as to emulsify the mercury. We found inconsistency in the readings of CO attendant on the variations in the length and severity of shaking, and established this technique after carefully controlled experiments.[1] After taking the apparatus off the shaking machine, the lower stopcock is opened and 1.5 cc. of pyrogallic acid solution (12 grams in 72 cc. of a 33 per cent NaOH solution) are put in the cup and run in a little at a time to absorb the oxygen. When the reading is constant the solution is trapped as completely as possible in the bulb below the lower stopcock, the graduated tube is rinsed clean with water, which also permits the reading of the bottom of the meniscus, previously impossible on account of the turbidity of the solution. That no leak has occurred during the procedure is verified by running in a little of the copper solution. This absorbs all but about 0.025 cc. of the gas. The analysis is duplicated. Previously we have been satisfied to obtain duplicate readings which vary by as much as 0.01 cc. With further experience they now rarely vary more than 0.005.

Discussion of the method of analyzing the blood. The accuracy of this method depends on the technique of the person working with the instrument. To check this we did a set of controls (table 1). About 2 cc. of blood are run into the 50 cc. chamber which is then evacuated and the blood shaken. By this procedure the oxygen in combination with the hemoglobin is removed. This may be run out of the machine at intervals. Within 20 minutes the blood is almost completely deoxidized. The blood is then trapped in the lower bulb and the 50 cc. chamber washed out. The connections are rinsed with CO gas and about 0.2 or 0.3 cc. of CO is introduced and washed in with a few drops of water to empty the bore of the stopcock and give a concave meniscus.

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[1] Since sending this paper to press, an article by Theo. K. Kruse in the American Journal of Physiology, Volume lv, number 2, page 289, has come to our notice. In conformity with his results, we find that the long shaking is unnecessary, if, after adding the potassium ferricyanide, and sealing, the solutions are allowed to stand in contact for ten minutes. The apparatus is then evacuated and shaken three minutes longer.

The blood is then released from the lower bulb into the upper chamber and mixed with the gas. With gentle shaking the CO is soon completely absorbed. Six cubic centimeters of the ammonia saponin and caprylic alcohol solution, freed of its gas content in a second Van Slyke instrument, are then poured into the cup and let in to the chamber. The routine analysis is made. As the figures in table 1 indicate, the amount of CO recovered corresponds (within the limit of error of our technique) to that introduced into the machine.

TABLE 2

Blood analyses performed immediately and after 24 hours both with and without oil

EXPERIMENT	SAMPLE DRAWN WITHOUT OIL		SAMPLE DRAWN UNDER OIL	
	At once	After 24 hours	At once	After 24 hours
	cc.	*cc.*	*cc.*	*cc.*
I	0.202	0.200	0.200	0.200
II	0.158	0.160	0.158	0.157
III	0.185	0.180	0.182	0.181
IV	0.238		0.240	
V	0.194	0.190	0.192	
VI	0.180	0.175		
VII	0.190	0.185		
VIII	0.140	0.140		
IX	0.260	0.260		
X	0.123	0.125		

Accuracy in the method of analyzing the blood is, however, useless if gas is lost either in handling the samples of blood or by escape from the blood stream through the lungs or into the tissues. It has been formerly customary to draw the blood under oil and take care that it should not be exposed to the air at any time because of diffusion of the gas. Table 2 shows the result of a series of experiments in which samples drawn under oil are compared with those drawn without oil, both when the samples are analyzed immediately and after an interval of 24 hours. It will be seen that the loss by diffusion from either sample even over a period of 24 hours is not appreciable.

Table 3 shows the result of a series of experiments to determine the interval of time, after removing the nosepiece, which may elapse before the analysis of the blood indicates an appreciable decrease in content of CO. Samples were drawn either while the hood was still on or within 1 or 2 minutes of removing it. Samples were then taken after 4, 7 and 10 minutes. Only the last shows a drop in the reading. We have, therefore, chosen 4 minutes as a routine length of time to allow complete, even distribution of the gas. Haldane and Smith (3) discuss the possible loss of gas by physical solution in the body fluids. The tension in the blood is so low (0.2 per cent, that supposing all body fluids to be saturated at this same tension the quantity of CO so bound is less than 1 cc.

TABLE 3

Readings of samples of blood drawn at varying intervals after inhalation of CO

DOG NUMBER	WEIGHT	CARBON MONOXIDE LIBERATED BY 2 CC. OF BLOOD			
		1 to 2 minutes	4 minutes	8 to 9 minutes	10 minutes
	kgm.	*cc.*	*cc.*	*cc.*	*cc.*
19–128	27.2	0.200	0.200	0.200	0.190
19–128	27.2	0.175	0.180	0.185	0.175
18–4	18.2	0.160	0.160	0.160	0.155
18–4	18.2	0.194	0.190	0.182	0.178
21–11	29.5	0.195	0.195	0.195	0.195
19–102	18.2	0.205	0.202		0.193
21–6	10.4	0.210	0.210		0.200
Average		0.191	0.191		0.184

Method of calculating the blood volume. To calculate the blood volume, first reduce the volume of gas inhaled to standard conditions by multiplying by the factor $(0.999\text{–}0.0046\ t) \times \frac{\text{barometer}}{760}$ where t is the temperature in degrees centigrade. From this must be subtracted the CO remaining in the respiratory chamber, as determined below.

The reading of the gas obtained from the blood analysis may be converted directly into cubic centimeters of CO per 100 cc. (volume per cent) of blood by using table 1 of Van Slyke's paper on oxygen (8). There is a small amount of nitrogen which is derived from the physically dissolved air in the blood and is read along with the CO after the pyrogallic acid has absorbed the oxygen. So to correct for this 1.2 must be subtracted from the volume per cent derived above as determined by Van Slyke (7). This may either be corrected for as stated, or eliminated as mentioned previously, by absorbing the CO with the copper solution and thus reading directly the true CO volume. Having now the total gas absorbed and the number of cubic centimeters of this recovered in 100 cc. of blood, the former divided by the latter gives the total blood volume. The hematocrit gives the proportion of cells to plasma, while from the weight of the dog the cubic centimeters of blood per kilogram body weight may be calculated. The reading of CO obtained in the Van Slyke apparatus is directly proportional to the cell per cent hematocrit and the blood volume is inversely proportional to the reading. It of course is obvious, therefore, that a hematocrit representative of the entire blood volume is not necessary, but it is necessary that the sample for the hematocrit and the sample to be analyzed should be taken at the same time so that the per cent of cells in each is the same. If this is the case the true measure of the body hemoglobin will be obtained, though the plasma volume and total volume will be inaccurate according as the hematocrit fails to be representative of the per cent cells in the whole blood.

Analysis of the gas remaining in the respiratory chamber. The principle of the method of analysis employed is to absorb the oxygen from a 100 cc. sample of the gas by means of pyrogallic acid solution. The remaining gas is then mixed with 2 cc. of blood, deoxidized as described before. This oxygen-free blood absorbs the CO which is then determined by the routine analysis of the blood sample. The technic in detail is as follows: A 100 cc. sample of the gas is drawn into a graduated tube of capacity slightly over 105 cc., one end of which is provided with a three-way stopcock, while the other end is connected with a leveling bulb. Mercury is used to adjust the pressure. The tube resembles a urea tube and is graduated only in 5 cc. intervals. Five cubic centimeters of pyrogallic acid are added through one arm of the three-way stopcock and the whole apparatus is shaken at short intervals to facilitate absorption of the oxygen. Meanwhile a little over 2 cc. of blood is deoxidized in the Van Slyke gas apparatus. In about one-half hour the reading in the tube has become constant. The two are connected with rubber tubing and the connections are filled with mercury. As much gas as the analyzer will hold, at atmospheric pressure, is run over and mixed with the blood. Gentle rotating to spread the blood over the walls of the chamber, with remixing of the gas in the tube and Van Slyke apparatus at 3 minute intervals, insures practically complete absorption of the CO within 45 minutes. The blood is then analyzed by the routine technic—the gas-free ammonia solution being introduced as above. An average of those analyses given in table 4, in which the per cent of residual O_2 is approximately 30 per cent, gives, with a calculated total chamber and lung volume of 1500 to 2000 cc. (depending on the size of the dog) approximately 2.00 cc. of unabsorbed CO. The total volume of lungs and respiratory chamber is estimated rather than measured, as by Gréhant and Quinquaud, as the correction would be at most less than 1 cc. of gas, which is a degree of precision the accuracy of the method does not permit.

The limited number of experiments so far performed suggests that the amount of CO remaining in the respiratory chamber is a factor of the per cent of O_2 found in the jar. Thus table 4 shows that the residual CO is doubled or tripled when the per cent of O_2 is between 40 and 50. The amount of O_2 introduced into the respiratory chamber is now regulated so as to constitute approximately 30 per cent of the total volume. Under these conditions the residual CO amounts to about 2 cc., which figure we now use in calculating the volume, as mentioned above.

This method is not the one first employed in the laboratory, nor is it identical to those described in the literature. It has finally been adopted because it is reasonably easy to carry out and may readily be controlled. Two series of controls were run. First, 100 cc. of air from the room or out of doors were analyzed in the same way. It was found that on an average 0.08 cc. of a gas, which was not absorbed by pyrogallic acid but was absorbed by the copper solution, was obtained. The gas was shown finally to be derived from the pyrogallic acid then in use. Subsequent experiments with other samples of pyrogallic acid gave at most 0.01 cc. of such gas. The second series of controls consisted in measuring in the analyzer a small quantity of CO, 0.2 to 0.3 cc., and

TABLE 4

Analyses of 100 cc. samples of gas from respiratory chamber taken immediately after blood volume determination

EXPERIMENT	DOG NUMBER	O_2 IN RESIDUAL GAS	CO IN 100 CC. GAS	TOTAL RESIDUAL CO
		per cent	*cc.*	*cc.*
I	21–30	20	0.060	1.00
II	21–10	45	0.340	5.61
III	19-102	50	0.317	5.68
IV	21–5	44	0.310	4.65
V	19–128	30	0.110	2.20
VI	18–4	30	0.110	2.03
VII	21-6	30	0.110	1.80

TABLE 5

A known amount of CO mixed with 100 cc. of air. Quantitative recovery of CO by method described

EXPERIMENT	CO MIXED WITH 100 CC. OF AIR	CO RECOVERED IN BLOOD MIXTURE
	cc.	*cc.*
I	0.15	0.140
II	0.21	0.205
III	0.16	0.185
IV	0.31	0.314
Average................	0.208	0.211

of running this into the tube with 100 cc. of air. This gas was then analyzed by the same procedure as in the case of the respiratory chamber air. The tabulated results in table 5 show that the CO is completely recovered by this procedure.

Hemorrhage control experiments. The following hemorrhage experiments were carried out as a control of the CO blood volume method. If the CO method is accurate it should be possible to determine the hemoglobin volume before and after unit bleedings of known amounts and show that the measured and calculated hemoglobin values compare with reasonable accuracy. In all these experiments the control determination was made the day previous to the hemorrhage. The second blood volume determination was done immediately following the hemorrhage. The determination of blood volume was made from 4 minutes to 1.5 hours following the completion of the hemorrhage, depending on the condition of the animal after the bleeding. In each instance the blood was drawn by means of a needle in the jugular vein into 1.6 per cent sodium oxalate, measured, and the hematocrit values determined from a 10 cc. sample. Anesthesia was not used in these experiments which cause no discomfort to the dogs except the puncture of the skin by the hypodermic needle.

TABLE 6

Hemorrhage experiment

Dog 19–98. Young, active male. Weight, 55 pounds

DATE 1920	TIME	RED CELLS HEMATOCRIT	BLOOD VOLUME		BLOOD REMOVED			CALCULATED RED CELL VOLUME
			Cells	Plasma	Total	Cells	Plasma	
		per cent	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
February 23	11:30 a.m.	55.9	974	768				
February 24	4:30 p.m.	49.4			624	308	316	
February 24	5:30 p.m.	42.2	678	928				666
February 25	4:30 p.m.	32.3			615	199	416	
February 25	5:15 p.m.	30.8	535	1202				467
February 26	2:30 p.m.	27.5	461	1216				
February 27	1:15 p.m.	25.7	452	1307				
February 28	12:00 m.	27.1	524	1408				
March 5	2:00 p.m.	34.0	833	1616				
March 9	3:30 p.m.	39.0	856	1338				

20 cc. whole blood drawn for each determination.

Table 6 presents a typical experiment on a normal dog which was bled large amounts on two successive days. About one-third the hemoglobin volume was removed in each bleeding. The last column gives the expected or calculated hemoglobin or red cell volume. It is noted that the calculated and measured red cell volumes following the first bleeding are practically identical, 666 cc. and 678 cc. respectively. Following the second large hemorrhage, we note the measured cell volume is 60 cc. greater than the calculated cell volume. On the following day the hemoglobin volume has fallen to the expected level and one is inclined to explain the 2nd day's figures as due in part to an error in technique. However, there are physiological variations in these figures which cannot be discussed at this time but may explain some of these fluctuations. Following the low level of the two days after the hemorrhages, we note a steady gain in hemoglobin volume. It may be noted that the amount of blood removed for each analysis (20 cc.) is allowed for in the bleeding periods and recorded figures. The calculated volume in each instance is obtained by subtracting from the normal cell volume the actual amount of red cells removed during the bleeding and experimental analysis.

TABLE 7

Hemorrhage experiment

Dog 20–37. Young, active, female. Weight, 22 pounds

DATE 1920	TIME	RED CELL HEMATOCRIT	BLOOD VOLUME		BLOOD REMOVED			CALCULATED RED CELL VOLUME
			Cells	Plasma	Total	Cells	Plasma	
		per cent	*cc*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
February 16	3:30 p.m.	50.8	**433**	420				
February 17	3:30 p.m.	50.3			250	126	124	
February 17	5:30 p.m.	39.9	**322**	483				**307**
February 18	4:00 p.m.	30.6	**310**	704				
February 19	4:15 p.m.	29.8	**310**	731				
February 20	2:30 p.m.	30.6	**302**	684				
March 12	7:50 a.m.	55.0	**418**	342				

20 cc. whole blood drawn for each determination.

Table 7 shows a single hemorrhage experiment. The calculated and measured red cell volume correspond very closely, 307 cc. and 322 cc. respectively. Following this bleeding there is not a prompt response nor any appreciable amount of hemoglobin constructed during the 3 days following the large hemorrhage. The daily bleeding of 20 cc. may have disturbed the normal body reaction tending to reproduce hemoglobin and red cells and we must assume that hemoglobin construction was practically stationary. In general we may say that following these large bleedings there was little evidence of very active reconstruction of red cells and hemoglobin during the first period of 2 to 5 days. It is more than probable that the daily bleedings of 20 cc. whole blood plus the CO inhalation actually inhibited the normal reaction which we believe is prompt and productive under normal conditions.

Table 8 shows an experiment very similar to that recorded in table 6. After the first bleeding the calculated and measured red cell volume correspond closely, the measured volume exceeding the calculated by 20 cc. After the second large bleeding we note again the excess of the measured volume over the calculated hemoglobin volume as 36 cc. This might be explained as a temporary emergency reserve reaction due to mobilization of hemoglobin normally retained in sluggish or isolated sinusoids of the spleen and red marrow. The reaction is similar in all these three experiments. Also there is a fall to the expected value on the days following the hemorrhages. The several days following the two bleedings in the experiment show periodic fluctuations in red cell volume but no significant rise in hemoglobin values. We hope to come back to this point again in subsequent publications.

TABLE 8

Hemorrhage experiment

Dog 19-66. Young, active male. Weight, 29 pounds

DATE 1920	TIME	RED CELL HEMATOCRIT	BLOOD VOLUME		BLOOD REMOVED			CALCULATED RED CELL VOLUME
			Cells	Plasma	Total	Cells	Plasma	
		per cent	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
February 2	4:30 p.m.	59.6	**573**	388				
February 3	3:00 p.m.	58.1			330	192	138	
February 3	3:14 p.m.	56.7	**403**	307				**383**
February 4	4:20 p.m.	35.5			330	117	213	
February 4	5:04 p.m.	34.9	**300**	560				**264**
February 5	3:30 p.m.	25.5	**261**	761				
February 6	3:30 p.m.	26.2	**324**	911				
February 9	12:15 p.m.	30.5	**300**	683				
February 12	3:00 p.m.	32.8	**318**	650				
March 11	2:30 p.m.	52.4	**528**	479				

20 cc. whole blood drawn for each determination.

Table 9 shows an experiment with two large bleedings on consecutive days. This experiment gives the measured values as *lower* than the calculated red cell volumes. We may explain this apparent exception to our other experiments as due to a slight error in the base line or normal hemoglobin values. The difference, however, is not great and the final hemoglobin volume of 179 cc. is very close to the calculated value of 184 cc. This dog, unlike the others, shows a distinct gain in hemoglobin volume in the few days after the hemorrhages.

The experiments in table 10 show how closely the cell volumes agree when the determinations are made on the same animal at different periods. The animals were all young healthy adults. Puppies show certain variations which are being studied at present and will be reported at some future time. In two instances here two determinations

TABLE 9

Hemorrhage experiment

Dog 20–34. Young, active male. Weight, 23 pounds

DATE 1920	TIME	RED CELL HEMATOCRIT	BLOOD VOLUME		BLOOD REMOVED			CALCULATED RED CELL VOLUME
			Cells	Plasma	Total	Cells	Plasma	
		per cent	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
April 13	3:30 p.m.	58.0	**434**	315				
April 14	3:00 p.m.	55.0			260	143	117	
April 14	3:10 p.m.	49.2	**265**	273				**291**
April 15	3:20 p.m.	41.0			260	107	153	
April 15	3:40 p.m.	35.9	**179**	319				**184**
April 16	2:00 p.m.	29.1	**233**	568				
April 20	2:30 p.m.	36.0	**292**	519				

20 cc. whole blood drawn for each determination.

TABLE 10

Blood volume determinations on normal dogs. CO method—red cell volume

DOG NUMBER	DATE	WEIGHT	HEMATOCRIT	RED CELLS	PLASMA	BLOOD PER 100 GRAMS	CELLS PER 100 GRAMS
		kgm.	*per cent*	*cc.*	*cc.*	*cc.*	*cc.*
19–66	December 29, 11:15 a.m.	13.2	63.4	**602**	347	7.21	**4.57**
19–66	December 29, 4:15 p.m.	13.2	60.3	**604**	355	7.28	**4.39**
19–66	February 2	13.2	59.6	**573**	388	7.30	**4.36**
19–137	December 10	12.9	50.6	**540**	528	8.25	**4.17**
19–137	December 11, 3:30 p.m.	12.9	53.4	**563**	491	8.14	**4.37**
19–137	December 11, 4:45 p.m.	12.9	48.9	**567**	593	8.97	**4.39**
19–137	December 16	12.9	50.8	**536**	520	8.16	**4.15**
20–37	February 16	10.0	50.8	**433**	420	8.54	**4.34**
20–37	March 12	10.4	55.0	**418**	342	7.28	**3.68**
21–5	August 6	6.6	46.3	**249**	289	8.16	**4.38**
21–5	August 9	7.0	43.7	**239**	308	7.77	**3.89**
21–5	August 17	7.2	43.3	**244**	320	7.89	**3.94**
21–5	August 18	6.6	43.0	**243**	323	8.60	**4.26**
21–5	September 28	6.9	52.3	**253**	231	6.99	**4.09**
21–5	October 18	7.0	52.4	**253**	230	6.86	**4.02**
21–5	October 21	7.2	50.3	**246**	254	6.84	**3.86**
Average				**454**	389	7.78	**4.20**

were made on the same animal during the same day. In both of these cases there was a slight residue of CO gas remaining in the blood when the second determination was carried out. This was corrected by making a CO analysis with a sample of blood drawn immediately before determining the second blood volume. The variation in cubic centimeters of blood per 100 grams of animal's body weight is due to the variation in the plasma volume determination. The cell volume varies within experimental limits. In other words, here again is evidence of a reasonably correct cell volume determination by the CO inhalation method as outlined above.

DISCUSSION

We wish to stress the fact that the carbon monoxide method gives a true measure of the pigment substances of the body which hold CO so tenaciously. These pigment substances include the hemoglobin of the circulating blood and probably that in the stagnant sinusoids of the spleen and red marrow. It is very probable that the muscle hemoglobin (myohematin) is also included in these pigment substances which dilute rapidly the inhaled carbon monoxide. We believe then that this CO method gives an accurate measure of this *body hemoglobin* but we fall into decided error when we *calculate* the total blood volume from the red cell hematocrit. The correctness of this calculation depends on the assumption that the blood hematocrit obtained from the jugular vein is representative of all parts of the body circulation, including arterioles and capillaries. We believe these figures obtained by *calculation* are *incorrect for the plasma volume* which is actually much larger than the hematocrit calculation would indicate.

In this paper we use the term *hemoglobin volume* to indicate the pigment substances which are concerned in the dilution of the inhaled carbon monoxide. It is evident that we do not believe that this figure represents only the actively circulating hemoglobin in the red cells but this circulating hemoglobin, of course, makes up the greatest part of the figure (90 to 95 per cent).

The hemorrhage experiments in which unit amounts of blood are removed, the body hemoglobin being measured before and after the single or double bleedings, give strong evidence of the accuracy of the method employed. The calculated and actual measured values correspond very closely. Moreover, repeated determinations on normal dogs under uniform conditions give figures which are quite uniform. All this indicates that the method of estimation is accurate provided we understand the fact that we are measuring only the *body hemoglobin* and *not the plasma volume*.

SUMMARY

The carbon monoxide method gives a reasonably accurate measure of the *body hemoglobin* and *hemoglobin volume*. We use these terms to indicate the total body pigment substances which fix the inhaled CO and bring about its dilution in the circulating blood. It is obvious that the true cell hemoglobin volume is slightly less than the total hemoglobin volume.

Repeated hemoglobin volume estimations on normal dogs give figures which are reasonably constant, allowing for certain expected physiological variations.

Hemoglobin volume estimations before and after unit hemorrhages give figures for the actual *measured* and indirectly *calculated* amounts which are in close accord.

In our opinion this method alone gives no accurate information concerning *plasma volume*.

Various controls of the several steps of this method, as used in our hands, are submitted. These controls establish this method as accurate at least within 5 per cent error. Further refinements and the use of packed red cells, especially in anemia, increase the accuracy of the method.

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BLOOD VOLUME STUDIES

VI. Plasma Volume as Determined by Hemoglobin Injection

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The hemoglobin method is exactly like the vital dye method and consists in the introduction intravenously of a known amount of a fresh hemoglobin solution. After a 4 minute period to permit of uniform distribution of the hemoglobin in the circulating blood a sample of blood is drawn into isotonic oxalate. From this plasma sample is determined colorimetrically the degree of dilution of the known hemoglobin introduced and the blood plasma volume is directly measured.

We wish to repeat that this method and many other similar methods give reasonably accurate figures for the blood *plasma volume* but we may not assume that the calculated red cell volume as determined by the hematocrit is also accurate. This point will be elaborated in the following paper.

All our experimental data point to a relatively *constant blood plasma volume* in adult dogs under uniform conditions. Estimated by the hemoglobin or dye methods the normal blood plasma volume of an adult healthy active dog is very close to 4.9 to 5.0 cc. per 100 gram body weight. There are normal variations due to age, physical condition and physiological activities which we hope to review in the near future. At this point we may refer to observations made in this laboratory by means of the dye method (3), (2), (7).

One need not be surprised at certain fluctuations in the plasma volume as determined by the hemoglobin or dye methods. On the other hand there is more reason for surprise at the constant figures obtained under certain conditions. When we contrast circulatory conditions in resting dogs with those of great muscular activity or digestive activity we may well wonder at the mechanism which shuts off one mass of body capillaries as another is opened to great activity. One can imagine that the greater part of spleen and red marrow sinusoids may at times be very incompletely washed with circulating plasma or again actively a part of the circulation. There is reason to believe that this constant circulating blood plasma is one factor in itself which is concerned with the partial isolation of one capillary field as another is opened for activity (digestion, for example). The *blood proteins* may be concerned in this adjustment complex and it has been pointed out in publications (4), (5) from this laboratory that the body will not tolerate great reductions in the concentration of the blood plasma proteins; further that the body can form these serum proteins only with difficulty and slowly. If the blood plasma proteins regulate circulating plasma fluctuations we may say that the body tissues which produce or replace the plasma proteins may take part in the regulation of circulating plasma volumes. We hope to supply experimental data which will help to solve these problems.

Some critics say that the dye method may be inaccurate because of the fact that a chemical substance foreign to the body is introduced into the blood stream. The very presence of this dye may modify circulatory conditions although admittedly there is no evidence of any cardio-vascular disturbance. To answer this objection hemoglobin is an ideal substance as it is not foreign to the blood stream and may be given intravenously in considerable amounts to dogs and man without the slightest clinical disturbance. Hemoglobin was first used in this way by Barratt and Yorke (1). The accuracy of the hemoglobin method is largely dependent on our knowledge that no hemolysis of red cells is caused at any step in the procedure. We have controlled this point in our experiments as given below. It is significant that the dye method and the hemoglobin method give identical figures for blood plasma volume.

Method. The general technical procedure in these experiments is identical with that previously described in work on blood plasma volume with vital dyes (3). The hemoglobin solution used in our work is prepared in the following manner: About 12 cc. of blood from the jugular vein of a dog are emptied into each of two or three 15 cc. centrifuge tubes containing about 2 cc. of 1.6 per cent oxalate solution. These tubes are then centrifuged for 30 minutes at 2500 revolutions per minute. After removing the plasma, 6 cc. of the packed red cells are mixed and shaken for 5 minutes with 30 cc. of distilled water. This makes a dilution of 1 cc. of packed cells in 6 cc. of fluid, which is a concentration at which the red cells are completely laked. This solution is clear and contains a negligible amount of stroma which will settle out on centrifugalization or on standing over night. In most of our experiments this hemoglobin solution is not centrifugalized but allowed to stand over night in the ice chest. Twenty-five cubic centimeters of the hemoglobin solution is the amount found to be satisfactory for determining the plasma volume of an average dog. More or less may be used, depending upon the size of the dog, and the individual taste in color readings.

In testing this method only normal adult dogs were used. A clean hypodermic needle is inserted into the jugular vein, and about 12 cc. of blood drawn in a clean syringe previously rinsed in 0.9 per cent saline. This sample of blood is then emptied carefully without bubble formation to prevent hemolysis, into a 15 cc. graduated hematocrit tube containing 2 cc. of 1.6 per cent sodium oxalate solution. The syringe is then rinsed in 0.9 saline and another sample of blood drawn and emptied into another oxalated hematocrit tube similar to the first. The blood and oxalate are mixed by inverting the tubes twice, which are then stoppered. Twenty-five cubic centimeters of hemoglobin solution, which has previously been measured into a porcelain dish using a calibrated pipette, are drawn up into a large syringe along with 5 to 10 cc. of 0.9 per cent saline used to wash out the porcelain dish. This solution is now injected slowly into the jugular vein and the hemoglobin rinsed out of the large syringe by withdrawing and reinjecting a little blood. The needle which has been in the vein during this process is allowed to remain, while about 10 cc. of saline are slowly injected to prevent clot formation in the needle. Exactly 4 minutes after injection of the hemoglobin solution a sample of blood is drawn in a clean syringe previously rinsed with 0.9 per cent saline, and emptied into a 15 cc. hematocrit tube containing 2 cc. of 1.6 per cent sodium oxalate. Immediately the syringe is rinsed in 0.9 per cent saline and a second sample is collected in a similar manner. Both the tubes are then inverted twice to insure thorough mixing, and stoppered. All four tubes are now centrifugalized at 2500 revolutions a minute for 30 minutes. The total contents and the number of cubic centimeters of cells are then noted. The red cell hematocrit normal values are computed from the control tubes.

The hemoglobin colored plasma is read in a Hellige's colorimeter against a standard prepared as follows:

1. One cubic centimeter of the hemoglobin solution from which 25 cc. were taken above for injection, is pipetted into a clean test tube to which is then added exactly 9 cc. of distilled water, thus making a 10 per cent hemoglobin solution.

2. Five cubic centimeters of the above 10 per cent solution are then diluted with 10 cc. of clear plasma obtained from the first two samples of blood drawn from the dog. Thus 0.5 cc. of the hemoglobin solution is diluted in 15 cc. of the standard.

Against this standard the colored unknown plasma is read and expressed in per cent. In the formula below let this reading be referred to as R. The number of cubic centimeters of hemoglobin solution injected into the dog is referred to as D, and the correction for the dilution by the oxalate present in the tinted plasma as C. Thus C expresses the ratio between the actual concentration of color in the plasma when diluted with 2 cc. of oxalate solution to the value when not so diluted.

The standard for color comparison contains 1 cc. of hemoglobin solution in 30 cc. of fluid.

Therefore D cc. of the hemoglobin solution (amount injected) will impart the same color intensity to 30 D cc. of fluid.

If, however, the color intensity is $\frac{R}{100\ C}$ (i.e., the colorimetric reading in per cent corrected for the dilution of the plasma by the oxalate solution).

Then, the number of cubic centimeters of fluids that have been tinted by D cc. of hemoglobin solution = $\frac{(30\ D)\ (100\ C)}{R}$ or, the *plasma volume* in cubic centimeters = $\frac{3000\ D\ C}{R}$.

Unless otherwise stated all experiments were done upon healthy dogs. A mixed diet of table scraps, including bones, meat, bread, potatoes, rice, etc., was given daily. Water was in the cages at all times.

Method control. If any hemolysis of red cells is produced by any of the steps of this method then the method has little if any value. Dogs' corpuscles are notoriously fragile and all workers in this field should control the method in their hands under local laboratory conditions. For this reason all specimens are drawn in duplicate and all readings of these duplicate specimens must check. Blood should be emptied down the sides of centrifuge tubes as air bubbles often cause slight hemolysis. For the same reason the last 1 to 2 cc. of blood in the syringe which may contain air bubbles should be discarded.

Our solution of hemoglobin is not made isotonic. Does this hypotonic solution by itself cause any hemolysis in the circulation? We controlled this step and others in the method by the injection of distilled water into the vein instead of the hemoglobin solution. In all other respects the blood samples, etc., were handled in the usual fashion described above. Two samples of blood were drawn into isotonic sodium oxalate after a period of 4 minutes following the injection of 25 cc. of distilled water. Six dogs injected in this way with 25 cc. of distilled water showed water-clear blood plasma obtained in the usual way. There was no hemolysis.

Other experiments in which larger amounts of distilled water were injected intravenously show that a very faint trace of hemolysis is observed only when the distilled water injected amounts to approximately 10 per cent of the total plasma volume. It is obvious therefore that we are within safe limits when hypotonic solutions in amounts less than 5 per cent of plasma volume are used for intravenous injection.

Experimental observations. One of the first things to be established for the hemoglobin method, as for the dye methods, is the curve of disappearance of the introduced hemoglobin. It will be noted that the hemoglobin leaves the circulation less promptly than the vital dyes but unlike the vital dyes is completely removed from the plasma within 18 hours. The body has other methods of dealing with hemoglobin in the circulating plasma as compared with vital dyes which persist in the blood stream for many days, depending in part upon the amount injected (2). For certain purposes this complete removal of the hemoglobin from the blood plasma within 18 hours is of especial importance and makes the hemoglobin method of distinct value for certain experimental work.

TABLE 11

Rate of elimination of hemoglobin solution from circulating blood plasma

DATE 1920	DOG NUMBER	WEIGHT	2 MINUTES	4 MINUTES	10 MINUTES	30 MINUTES	2 HOURS
		kgm.					
February 2	20–80	15.91	100	101	96	92	74
February 17	20–80	18.17	100	99	96	86	75
February 18	20–80	17.95	100	100	94	86	60
February 19	18–4	20.21	100	102	97	96	78
February 20	18–4	20.11	100	100	99	91	
February 25	18–4	20.45	100	98	94	92	78
Average................................				100	96	91	73

Table 11 gives the figures for the decrease in concentration of the injected hemoglobin in the circulating plasma. In all experiments 25 cc. of the standard hemoglobin solution were injected into the jugular vein. The samples removed at the end of 2 minutes are figured as 100 per cent—all samples in duplicate. It is clear that the hemoglobin remains in the blood stream with a minimal removal during the first 10 minutes. We think it is safe to assume that a 4-minute sample will give values which should be within 2 to 3 per cent of the true dilution if there was absolutely no escape of hemoglobin in the circulation. We consider that this figure is within the accuracy of the colorimetric determination.

After 1 to 2 hours the plasma samples take on a slightly brownish tint which makes accurate color comparisons difficult. This is undoubtedly due to the change of circulating hemoglobin into bile pigment substances (6), which is known to take place rapidly in dogs. This reaction is a recognized body function and makes for a complete and rapid removal of the injected hemoglobin, so that the plasma figures after 18 hours are zero. It is possible by the hemoglobin method to repeat such blood volume observations at relatively short periods without the troublesome complication of a residual plasma dye.

Table 12 includes many observations on young adult, active, healthy dogs. Several observations on the same animal under different dates are recorded. There are considerable variations in body weight and plasma volume. The dogs were all on a diet of mixed table scraps but no effort was made to insure a constant food intake and body weight in these experiments. The average for this group of dogs is 51.8 cc. plasma per kilo body weight. Calculated blood volume on the basis of a 50 per cent hematocrit introduces the error already referred to. These figures correspond to those recorded in this laboratory with the brilliant vital red method.

TABLE 12

Blood plasma determination on normal dogs

DATE 1920	DOG NUMBER	WEIGHT	HEMATOCRIT RED CELLS	PLASMA VOLUME	CELL VOLUME	TOTAL BLOOD VOLUME	BLOOD PER KILOGRAM	PLASMA PER KILOGRAM
		kgm.	*per cent*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
January 22	20–80	17.85	48.6	921	871	1792	100.4	51.6
January 30	20–80	16.81	47.4	918	855	1773	105.5	54.6
February 17	20–80	18.17	45.8	1057	895	1952	107.4	58.1
April 7	20–73	16.36	55.8	741	934	1675	102.4	45.3
February 9	18–4	19.88	56.7	928	1218	2146	107.9	46.7
February 19	18–4	20.21	55.4	993	1161	2154	106.5	49.1
February 20	18–4	20.11	49.1	1089	987	2076	103.2	54.1
February 25	18–4	20.45	47.0	1113	1001	2114	103.3	54.4
March 23	18–4	19.08	55.4	868	1076	1944	101.9	45.5
April 19	18–4	18.40	48.6	1082	1020	2102	114.2	58.8
August 27	19–102	16.82	50.7	876	900	1776	105.6	52.1
Average		18.55	50.9	962	992	1954	105.3	51.8

Table 13 is very much like the preceding table but these dogs are all much heavier. It is a constant observation in this laboratory that these large, heavy, quiet dogs give lower plasma volumes than do the active, muscular dogs of smaller size. A part of this plasma volume decrease undoubtedly is due to the increase in fat which is less rich in capillaries than other body tissues like muscle. The plasma volume, therefore, may be expected to decrease per kilo as a dog puts on fat and becomes less active. Our highest blood volume figures per kilo are recorded in lean, very active, young adult dogs. The blood plasma figures are apt to be more constant in the larger dogs—see dog 19-128, table

13. Compare the average plasma volume per kilo = 51.8 cc. for the active dogs, and the plasma volume per kilo = 41.0 cc. for the large, fat dogs (table 13).

TABLE 13

Blood plasma determination on normal large dogs

DATE 1920	DOG NUMBER	WEIGHT	HEMATOCRIT RED CELLS	PLASMA VOLUME	CELL VOLUME	TOTAL BLOOD VOLUME	BLOOD PER KILOGRAM	PLASMA PER KILOGRAM
		kgm.	*per cent*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
March 17	19–128	28.17	55.5	**1064**	1339	2403	85.4	**37.7**
March 25	19–128	27.85	56.3	**1099**	1416	2515	90.3	**39.4**
April 7	19–128	28.52	55.5	**1080**	1349	2429	85.1	**37.8**
April 29	19–128	27.40	57.2	**1136**	1518	2654	96.8	**41.4**
June 7	19–128	26.70	60.5	**1143**	1749	2892	108.3	**42.8**
July 13	19–128	27.70	62.5	**1122**	1872	2994	108.1	**40.5**
July 20	19–128	27.50	61.6	**1137**	1824	2961	107.7	**41.3**
April 14	17–160	26.92	60.5	**1143**	1752	2895	107.4	**42.4**
June 24	17–160	24.18	50.4	**1159**	1179	2338	96.7	**47.8**
June 21	18–60	24.10	52.9	**976**	1095	2071	86.0	**40.5**
August 20	17–154	23.20	57.8	**995**	1345	2340	100.8	**42.8**
August 27	21–11	29.21	59.1	**1149**	1661	2810	96.2	**39.3**
Average		26.78	57.4	**1099**	1509	2608	97.4	**41.0**

TABLE 14

Daily blood plasma determination

Experiment A. Dog 18–4. Wire-haired mongrel. Lean and active female

DATE 1920	WEIGHT	HEMATOCRIT RED CELLS	PLASMA VOLUME	CELL VOLUME	TOTAL BLOOD VOLUME	BLOOD PER KILOGRAM	PLASMA PER KILOGRAM
	kgm.	*per cent*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
June 14	19.6	50.2	**1057**	1068	2125	108.7	**53.9**
June 15	19.6	48.0	**1100**	1053	2153	110.1	**56.1**
June 16	19.6	48.0	**1084**	1003	2087	106.8	**55.3**
June 17	19.6	45.8	**1220**	1032	2252	115.1	**62.2**
Experiment B. Dog 17–160. Large mongrel bull. Fat and inactive male							
June 14	25.3	53.6	**1120**	1294	2414	95.9	**44.2**
June 15	25.2	48.9	**1138**	1100	2238	88.8	**45.2**
June 16	25.1	48.5	**1203**	1134	2337	93.2	**47.9**
June 17	24.6	52.1	**1163**	1265	2428	98.9	**47.2**

Table 14 gives observations on two normal dogs with daily blood plasma determinations for 4 days. The dogs were fed the usual mixed diet in the afternoon after the blood volume determinations had been completed. They had access to water at all times. The lighter an more active dog has a higher blood plasma volume per kilo and shows the greater daily fluctuations. These daily bleedings of 20 to 30 cc. cause a slight upset in the red blood cell hematocrit values and may be in part responsible for the variations in plasma volume figures.

SUMMARY

The *hemoglobin method* gives a reasonably accurate measure of the *circulating blood plasma.* Fresh standard hemoglobin solutions can be prepared easily and when used with sufficient care will give uniform figures which are identical with those obtained by the common vital dyes used in blood volume work. The hemoglobin is completely removed from the blood within 18 hours, which may be of advantage in certain experiments.

Observations here recorded show that large, fat dogs present a plasma volume per kilo which is uniformly lower than the figures for lean, active, adult dogs.

In our opinion this method gives no direct information concerning red cell or hemoglobin volume.

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BLOOD VOLUME STUDIES

VII. Comparative Values of Welcker, Carbon Monoxide and Dye Methods for Blood Volume Determinations. Accurate Estimation of Absolute Blood Volume

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A review of the many blood volume methods and a consideration of the many apparent contradictions in these blood volume figures made it clear to us that a detailed study of these various methods was desirable. We hope to throw some light on many of these vexed questions so that finally we may come to understand the factors which regulate the volume of the circulating blood and recognize the limitations of the various methods. We submit the data of these experiments with the conviction that these figures obtained under uniform conditions, using the same animals and a controlled technic, are of greater value than the data to be obtained in the literature. The methods of different investigators are often incompletely described and may present gross errors—for example, the use of hypotonic oxalate solutions or dry powdered oxalate to prevent blood coagulation may introduce errors into the plasma volume figures which may exceed 10 per cent (1).

In brief, it is our conviction that the *dye or hemoglobin methods give the true plasma volume* (4.8 cc. per 100 grams of body weight), that the *carbon monoxide method gives the true body hemoglobin volume* (4.2 cc. R. B. C. per 100 grams body weight), and finally that the true blood volume is the sum of these two figures (9 cc. per 100 grams of body weight). Hematocrit figures introduce errors when used to calculate the plasma or red cell volume because of the assumption that the ratio of cells and plasma is a constant for all parts of the circulation. This error may become still larger in abnormal conditions where the ratio of cells of plasma may fluctuate more than normal in different areas of the circulation.

For several years these blood volume problems have been of interest to a considerable number of workers in this laboratory. These studies developed as a part of an investigation into the problem of hemoglobin regeneration after simple anemia. Some of these experiments have been published (2). In the course of this work certain defects in the blood volume method of Keith, Rowntree and Geraghty (3) were discovered and modifications were proposed by Hooper, Smith, Belt and Whipple (1). A variety of dye substances was tested by Dawson, Evans and Whipple (4). An acacia blood plasma volume method was described by McQuarrie and Davis (5) and certain advantages of this method pointed out. This critical study by many different workers of these various methods, their limitations and advantages, has been of great value in the development of an accurate and controlled technic.

In all of these methods the same principle is employed, viz., a known amount of the solution (dye, hemoglobin or acacia) is injected intravenously and adequate time is allowed for the complete admixture of this substance with the blood. At the end of this period a sample of blood is withdrawn into an isotonic oxalate solution and centrifugalized to remove the blood cells. The concentration of the injected substance in the supernatant plasma is then determined. It is now easy to compute the volume of plasma required to dilute to this degree the total mass of the substance injected. The original articles should be consulted for details of these methods. We wish to emphasize the fact that in theory all of these methods rest upon the same basis, viz., dilution of the injected substance by the blood *plasma*. For this reason these methods should be termed *plasma volume methods* rather than *blood volume methods*. A fact of equal importance is that these methods agree in showing that in normal adult dogs there are about 4.8 cc. of plasma per 100 grams of body weight.

The carbon monoxide method for the determination of blood volume has been the subject of study by Arnold, Carrier, Smith and Whipple (6). In its essential features their method is similar to that of Salvesen (7). They have carried out many controls on the method and have suggested some alterations in the method of Salvesen. The details of the method need not be repeated. In brief, the animal is forced to breathe into an air-tight but elastic chamber containing oxygen together with a measured amount of carbon monoxide. The carbon monoxide is drawn into the lungs along with the oxygen where it is absorbed almost quantitatively by the hemoglobin of the blood which passes continuously through the vessels of the lungs. The measured amount of carbon monoxide is thus transferred from the respiratory chamber to the hemoglobin of the circulating blood. A sample of blood is now drawn and its carbon monoxide content determined. The carbon monoxide concentration in this sample furnishes an index of the total quantity of hemoglobin in the body. It will be noted that whereas the other methods mentioned above indicate the *volume of plasma*, the carbon monoxide is taken up into the hemoglobin and thereby furnishes us with an index of the *total body hemoglobin.*

For purposes of further comparison of methods we have used a modification of the direct blood volume method first employed in 1854 by Welcker (8). The method consists essentially in determining the amount of hemoglobin which can be washed out of the animal body. Various modifications and improvements of Welcker's method have been made in the last 50 years. Some of these changes have made possible the more complete removal of the hemoglobin from the body. Others involve the use of the more recently devised methods of estimating the amount of hemoglobin once it is washed out of the body. The method as used by us is described in detail below By means of this method, as in the carbon monoxide method, the blood constituent primarily measured is the hemoglobin. With certain qualifications to be mentioned later, either of these two methods enables one to determine fairly accurately the *total red blood cell volume*, for the hemoglobin of the circulating blood is normally confined entirely to the red blood cells and for each animal there is a definite ratio between the hemoglobin and the red blood cells. These two methods should be termed *body hemoglobin* or *red blood cell volume methods.*

In the experiments to be presented below, blood volume determinations were carried out on each of a series of normal dogs, first by the dye method, shortly thereafter by the carbon monoxide method, which was in turn followed by a determination by a modification of the Welcker method. In this way an excellent opportunity is afforded to study and compare these three methods under practically identical conditions of experimentation.

Method. Adult dogs maintained on a mixed diet were used in all experiments. All were sound, healthy animals unless otherwise noted. Free access to water obtained at all times. Food was withheld for a period of about 12 hours prior to the carrying out of the three blood volume determinations. This was done in order that the plasma might be clear and free from fat.

In carrying out the dye method the procedure previously outlined by Hooper, Smith, Belt and Whipple (1) was followed in all respects. Following this, usually within a few minutes, a determination by the carbon monoxide method as outlined by Arnold, Carrier, Smith and Whipple (6) was carried out. For each of these determinations approximately 20 cc. of blood were drawn. Preparations were now made for the determination by the third method, i.e., the modified Welcker method. The animal was placed under complete ether anesthesia. A cannula was inserted into one of the larger arteries (carotid or femoral) in such a manner that blood could be withdrawn at will. The animal was bled at a moderately rapid rate from this artery. In order to prolong life and permit the heart to pump out a major portion of the circulating blood cells, warm saline was injected into the vascular system through a cannula inserted into one of the larger veins. In all cases the saline was thus infused into either the femoral or external jugular vein. The fluid was injected at a rate approximately equal to that at which the blood was being withdrawn from the artery. In most cases the heart's action ceased after this exchange of saline for blood had been continued for about 20 minutes.

In order to wash from the vascular system as completely as possible the remaining traces of blood, the inflow cannula was changed and the saline was forced under pressure into the arterial system. The fluid thus artificially forced to circulate was in great part recovered from the right side of the heart by means of a cannula inserted and tied into the right auricular appendage in such a way as to drain the fluid collecting in the right auricle. In several of the earlier experiments a part of the saline forced into the arterial system found its way back past the aortic valves into the pulmonary circuit, and forcing its way into the bronchi escaped through the nose. In subsequent experiments this was prevented by clamping a heavy hemostat about the aorta just distal to the aortic cusps during the period of artificial perfusion. The perfusate was thus prevented from backing up into the pulmonary circulation.

During the period of perfusion the inflow of saline was occasionally interrupted for a number of seconds, for the work of Larson and Bell (9) indicates that intermittent perfusion is more efficacious in washing blood cells out of the vascular system than is a steady continuous flow. To further aid in adequately washing out of the smaller vessels general massage of the animal body was practiced. In most cases the artificial perfusion was continued for about 1½ hour. The fluid recovered during the last part of this period appeared almost as clear as water and on centrifugalization such samples were found to contain very few blood cells indeed. At autopsy only scattered traces of macroscopic blood could be made out. These findings are described more in detail in individual experiments. The relative freedom of the vessels from blood as shown by microscopic study of stained sections will also be shown later.

All samples of blood recovered from the animal were received into oxalate to prevent clotting. The blood withdrawn before the death of the animal was collected separately in one container. The blood collected by artificial perfusion was also collected separately, usually in two portions, the last portion being of relatively large volume (5 to 15 liters) but containing as a rule less than 10 per cent of the total hemoglobin recovered from the animals. Each sample thus collected was measured and its hemoglobin content determined by the method of Palmer (10). The total number of grams of hemoglobin removed was thus ascertained.

In spite of the prolonged artificial perfusion a certain small part of the total hemoglobin remains behind in the body. In order to measure the total amount of the pigment which perfusion failed to remove we have followed the practice of grinding up and extracting from the various organs and tissues their content of pigment. The skin in all cases was stripped off and being almost free of pigment was discarded. The skeletal muscles together with the fascia were dissected free from the skeletal system, ground up finely in a meat grinder, mixed with water and stirred frequently for a period of about 6 hours. The fluid and particles of muscle were separated in a meat press. A sample of the fluid obtained was further freed of debris by centrifugalization, after which its hemoglobin content was estimated. The parenchymatous organs were separately and individually treated in a similar manner. The gall-bladder with its contents was previously dissected free and discarded. The bile and fecal matter were carefully washed out of the gastro-intestinal tract before this tissue was ground up for extraction. The bones were broken up finely with a hatchet before their extraction was attempted. It is recognized that only a part of the total pigment thus extracted is true red blood cell pigment. Another portion is extravascular pigment, particularly muscle hemoglobin. For this reason the organ extracts are charted separately in all tables. The question as to how much of this pigment can properly be considered to be blood pigment will be discussed later.

The quantity of *blood hemoglobin* recovered by bleeding and perfusion is contained wholly within the red blood cells washed out. Knowing the total number of grams of hemoglobin, the total number of cubic centimeters of red blood cells can be learned. To do this the hemoglobin content of a given amount of red blood cells is determined by reading the hemoglobin content of an initial sample of blood from the same animal and taking into account the ratio of red blood cells to plasma (hematocrit). These figures give us a factor by which the total hemoglobin washed out may be multipled in order to show the total volume of red blood cells recovered. This same factor may be used to convert the hemoglobin figure into red blood cell volume in the case of the carbon monoxide method.

The hemoglobin and red blood cell volume figures are thus given in a very direct and relatively trustworthy manner by the carbon monoxide method or Welcker method. In like manner the dye method being essentially a plasma volume method indicates with fair accuracy the total volume of plasma. In tabulating our results we have placed in bold type those values for whose determination each method is especially adapted. This for the dye method includes plasma volume; for the carbon monoxide or Welcker methods, the total hemoglobin and red blood cell volume. All other figures tabulated are derived indirectly from these on the basis of the hematocrit. As will be explained later, we have no assurance that the sample of blood centrifugalized (hematocrit) is representative of the entire blood. The figures based on the hematocrit are therefore open to criticism. Our method of tabulation enables one to distinguish these figures from those which are not open to this criticism and are placed in bold type.

Experimental observations. The general plan of experimentation has already been outlined. The results of 14 experiments are presented below. Several others which were incomplete in some important respect are omitted. Experiment 339 may be regarded as a typical experiment. The procedure followed is presented in detail as illustrative of the method used in all other experiments. In this experiment as in most of the others, glucose was added to the saline infused. Our experience indicates that if this be done the heart continues to beat for a longer time and a larger proportion of the blood is expelled before death occurs. This is to be desired because under conditions of artificial perfusion of dying tissues the vascular conditions are altered and circulation in the more remote parts of the vascular tree is sluggish. In addition the phenomenon of intravascular clotting following death may in some cases prove to be a disturbing factor. However, if artificial perfusion is relied upon to remove only the last traces of blood, such disturbing factors are of relatively slight importance. In this experiment the perfusion was continued for nearly 2 hours. During the greater part of this time the perfusate recovered was but slightly blood-tinged. During the last 30 minutes it was almost as clear and colorless as water. If 15 cc. of such a sample be centrifugalized only a very small layer of blood cells will be found in the bottom of the tube. In all 8 grams of hemoglobin were recovered through artificial perfusion, whereas 184 grams in all were recovered through bleeding and perfusion combined. Thus only 4.3 per cent of the total was recovered by perfusion.

Experiment 339. Dog 20–92. Adult male mongrel terrier, in good condition. Weight, 15.45 kgm. Blood volume determination by dye method at 7:37 a.m.: by the carbon monoxide method at 7:41 a.m. Ether anesthesia at 9:40 a.m. in preparation for carrying out the modified Welcker determination. A cannula was tied into the carotid artery. Bleeding from this cannula was commenced at 9:45 a.m. Simultaneously and at approximately the same rate Locke's solution containing 1 per cent glucose and warmed to body temperature was injected through a cannula into the external jugular vein. About 3500 cc. of this fluid were injected within a space of about 20 minutes. In the same period 3430 cc. of blood were withdrawn from the carotid artery. The heart continued to beat until nearly the end of this period at which time the blood flowing out was almost colorless. In order to wash from the vascular system as completely as possible the remaining traces of blood, saline was forced under pressure into the arterial system by way of the carotid. The fluid thus artificially forced to circulate was recovered from the right side of the heart by means of a cannula inserted through the right auricular appendage into the right auricle of the heart. During the period of artificial perfusion the inflow of saline was occasionally interrupted for a period of a minute or so. To further aid the perfusate in reaching and adequately washing out all of the smaller vessels, general massage of the animal body was practiced. The perfusion was discontinued at 11:10 a.m. after having been in effect nearly 2 hours.

At autopsy the peritoneal and thoracic cavities contain a small amount of clear blood-tinged transudate. No blood is found in the cavities of the heart.

TABLE 15

Experiment 339. Dog. 15.45 kgm. Hematocrit 50.1 per cent red blood cells

	TOTAL HEMOGLOBIN	TOTAL R. B. C.	BLOOD	PLASMA	PER 100 GRAMS BODY WEIGHT			
					Hemoglobin	R. B. C.	Blood	Plasma
	grams	*cc.*	*cc.*	*cc.*	*grams*	*cc.*	*cc.*	*cc.*
Dye method	277.0	857.0	1710.0	838.0	1.79	5.55	11.07	5.42
Carbon monoxide method	223.0	690.0	1378.0	675.0	1.44	4.47	8.92	4.37
Welcker method								
Total bleedings	184.0	568.0	1133.0	555.0	1.19	3.68	7.33	3.59
Extracts								
Heart	0.23	0.70	1.40	0.69	0.0015	0.0045	0.0091	0.0045
Spleen	0.26	0.80	1.60	0.78	0.0017	0.0052	0.0104	0.0054
Skeletal muscles	22.8	71.0	141.0	69.0	0.147	0.459	0.913	0.447
Bones	3.32	10.3	21.0	10.0	0.021	0.067	0.133	0.065
Total extracts	26.6	83.0	165.0	80.0	0.171	0.536	1.066	0.522
Total (including extracts)	211.0	651.0	1298.0	635.0	1.36	4.22	8.40	4.11

Lungs, liver, kidneys, gastro-intestinal tissues contain only trace of hemoglobin.

The myocardium is pale brown. The lungs are crepitant throughout, are pale grey and quite bloodless. The spleen appears quite normal. The liver is uniformly brown. The lobulation is very indistinct. No blood visible anywhere. The mucosa of the esophagus is pink. The entire gastro-intestinal tract appears quite bloodless. The pancreas, kidneys and pelvic organs contain no macroscopic blood whatsoever. The bone marrow (femur, ribs) is of a moderate bright red color.

In microscopic sections no red blood cells are seen in the heart, lungs or liver, and only an occasional one can be found in the pancreas. The spleen is quite rich in red blood cells; none whatsoever are found in the kidneys or gastro-intestinal tract. No red blood cells could be found in the skeletal muscle even after prolonged search.

The hemoglobin content of the samples recovered was now determined. From the carotid artery 3430 cc. of blood were recovered before the death of the animal. This was found by the method of Palmer to contain 167 grams of hemoglobin. To this must be added 9 grams of hemoglobin which were contained in the 55.6 cc. of blood drawn as samples for the dye and carbon monoxide method. A total ot 11,400 cc. was recovered during the period of artificial perfusion. In this there were 8 grams of hemoglobin. The sum of these quantities (184 grams) is entered in the table under the heading of "total bleedings." (See table 15.)

The skeletal muscles were stripped of and ground up finely in a meat grinder. Total weight, 6920 grams. This mass of ground muscle was thoroughly mixed up and to 500 grams 1500 cc. of distilled water were added. This was frequently and vigorously stirred for a period of several hours. By use of the meat press 1350 cc. of hemoglobin-tinged liquid were recovered. It is certain that there was more fluid which could not be forced out with the press. By a more powerful press we could undoubtedly have removed at least 1500 cc. (i.e., an amount corresponding to the quantity of water added for extraction). A sample of this fluid was further clarified by centrifugalization (30 minutes at 3000 revolutions a minute). To 100 cc. of this fluid 0.4 cc. of concentrated ammonia solution was added. Carbon monoxide was bubbled through and the solution was compared in a Duboscq colorimeter with a standard prepared as in the method of Palmer. This extract fluid was found to contain 0.11 gram of hemoglobin per 100 cc. At this rate 1500 cc. would contain 1.65 grams of hemoglobin. Had the entire 6920 grams of skeletal muscle been extracted instead of only 500 grams we should have recovered 22.8 grams of hemoglobin. The other organs and tissues were treated in the same manner. The bones were broken up finely with a hatchet before being extracted. In all cases the macerated tissues remaining behind in the meat press were quite pale, thus indicating that the extraction of hemoglobin had been quite complete.

A sample of blood drawn from the dog at 7:32 a.m. contained 50.1 per cent red blood cells as was shown by a thorough centrifugalization of a 10 cc. sample of blood drawn into a 15 cc. hematocrit tube containing 2 cc. of isotonic sodium oxalate (1.6 per cent (1)). Another sample of whole blood drawn at the same time was found to contain 16.20 grams of hemoglobin per 100 cc. Since the red blood cells constitute but 50.1 per cent of the volume of this blood it is easily seen that 100 cc. of packed cells would contain nearly twice this amount—32.3 grams per 100 cc., to be exact. This is equivalennt to saying that 1 gram of hemoglobin is contained in 3.09 cc. of red blood cells. At this rate 184 grams of hemoglobin (the amount in the "total bleedings") represent 568 cc. of red blood cells. Since there are 100 parts of blood for every 50.1 parts of red blood cells (as shown by the hematocrit) it follows that 568 cc. of red blood cells represent the quantity of red blood cells contained in 1133 cc. of blood. The difference between 1133 and 586 represents the volume of plasma plus that of the white blood cells. In this experiment the white blood cells comprised about 0.9 per cent of the total blood, as was shown by the hematocrit. The red blood cells plus the white blood cells, then, comprise 51.0 per cent of the blood. The other 49 per cent is plasma. Forty-nine per cent of 1133 is 555. This represents the total plasma in the "total bleedings." The organ extracts may be figured in the same manner with the results shown in table 15.

In the total extracts 26.6 grams more of hemoglobin were recovered. We are convinced that for practical purposes the pigment is completely extracted by our technic. A second extraction of the macerated bones was attempted in experiment 339, but as is noted in the protocol very little additional hemoglobin was dissolved out. Ordinarily a single extraction of finely macerated skeletal muscle leaves the muscle debris quite pale. Apparently by far the greater part of the pigment is removed. A second extraction of much muscle debris was made in a number of cases. A typical result is shown in experiment 349, table 16. In this case 15.9 grams were removed by the first extraction and but 2.0 grams by the second extraction. After the second extraction the muscle tissue was almost perfectly white. Similar extractions made in other organs and tissues show essentially identical results. Repeated extraction of such organs and tissues show that by far the greater portion of the pigment is removed by the first extraction, provided that to the macerated tissue at least an equal part of water be added for purposes of extraction.

Experiment 349. Dog 19–71. Adult male mongrel shepherd. For several weeks has been somewhat less active than normal and is somewhat undernourished but on the whole is in fairly good condition. Weight, 16.36 kgm. Blood volume determination by dye method at 8:40 a.m.; by the carbon monoxide method at 9:01 a.m. Bleeding and perfusion under ether anesthesia in the usual manner begun at 9:20 a.m. Warm 0.9 per cent saline to which was added 1 per cent of glucose was used for infusion. Following death of the animal the tissues were perfused with plain 0.9 per cent saline warmed to body temperature. A total of 24,580 cc. of blood-containing perfusion fluid was recovered.

At autopsy the thoracic organs appear to be almost perfectly free of blood. The spleen is normal in size and appearance. The liver is dark brown, the lobulation is distinct and no blood can be seen even at the centers of the lobules. The stomach appears to be bloodless. The mucosa of the upper part of the small intestine is faintly pink. The tips of some of the rugae of the mucosa of the large intestine are reddened. The pancreas, kidneys and pelvic organs are free of macroscopic blood. The bone marrow is bright red.

Microscopically but few red blood cells are seen in sections of myocardium. The lung contains a moderate number. As usual the spleen is quite rich in blood cells. A few are seen in the vessels in the hepatic spaces. Sections of pancreas are free of blood. A moderate number of red blood cells is found in the mucosa of the stomach and many in the mucosa of the small intestine. On the other hand sections of the large intestine and kidney are free of red blood cells. An occasional red blood cell is to be found in sections of skeletal muscle.

From the carotid artery 3580 cc. of blood were recovered before the death of the animal. This was found to conatin 158 grams of hemoglobin. In addition to this 11 grams of hemoglobin were contained in the 83 cc. of blood drawn as samples. A total of 21,000 cc. of perfusate recovered during the period of artificial perfusion contained 22 grams of hemoglobin. The skeletal muscles (7136 grams) were ground up, mixed thoroughly and a 200-gram sample extracted in the usual manner with 400 cc. of water. After pressing out the fluid the muscle debris was quite pale. To this debris 200 cc. of water were added and the mixture frequently and thoroughly mixed for several hours. Had the entire mass of skeletal muscle been extracted instead of only 200 grams we should have obtained by the first extraction 15.9 grams of hemoglobin and only 2.0 grams by the second extraction.

The other organs were extracted in the usual manner. By the first extraction the bones yielded 4.8 grams of hemoglobin; by the second extraction, 0.8 gram.

TABLE 16

Experiment 349. Dog; 16.36 kgm. Hematocrit 46.4 per cent red blood cells

	TOTAL HEMOGLOBIN	TOTAL R. B. C.	BLOOD	PLASMA	PER 100 GRAMS BODY WEIGHT			
					Hemoglobin	R. B. C.	Blood	Plasma
	grams	*cc.*	*cc.*	*cc.*	*grams*	*cc.*	*cc.*	*cc.*
Dye method	218.0	822.0	1772.0	932.0	1.33	5.02	10.83	5.69
Carbon monoxide method	209.0	729.0	1571.0	826.0	1.28	4.46	9.60	5 05
Welcker method								
Total bleedings	191.0	664.0	1430.0	752.0	1.17	4.06	8.74	4.60
Extracts								
Heart	0.24	0.84	1.81	0.95	0.0015	0.0051	0.011	0.0058
Spleen	0.13	0.46	1.00	0.53	0.0008	0 0028	0.0061	0.0032
Skeletal muscles	15.9	55.0	119.0	63.0	0.097	0.34	0.73	0.39
Bones	4.8	17.0	36.0	19.0	0.029	0.10	0.22	0.12
Total extracts	21.1	73.0	158.0	83.0	0.128	0.45	0.97	0.52
Total (including extracts)	212.0	737.0	1588.0	835.0	1.30	4.51	9.71	5.12

Lungs, liver, kidneys and gastro-intestinal tissue contain only traces of hemoglobin.

Additional evidence of the readiness with which hemoglobin can be separated from macerated tissues is furnished by an experiment in which we added a known amount of hemoglobin (in the form of blood) to a weighed sample of macerated skeletal muscle. The mixture was thoroughly stirred after which its hemoglobin was extracted in the usual manner. Analysis showed that we recovered by a single extraction practically all of the added hemoglobin as well as the quantity of pigment which the muscle was known to contain before the artificial addition of hemoglobin.

The freedom of the perfused tissues from red blood cells as shown by microscopic study is well illustrated in experiment 339. Almost complete absence of red blood cells in all sections was noted. In sections from normal unperfused dogs many red blood cells are to be seen. Our experience has been that as a rule macroscopic appearance of the tissues as regards its blood content is well borne out by the microscopic findings. Experiment 351 also serves to illustrate this point. This experiment represents one in which we were particularly *unsuccessful* in perfusing the organs free of blood. The mucosa of the small intestine was brick-red in color and the abundance of red blood cells was quite strikingly shown in microscopic sections of this tissue. The large intestines were free of macroscopic blood and in confirmation no red blood cells whatever could be found microscopically. In this connection it will be recalled that in experiment 339 the absence of macroscopic blood in the entire gastro-intestinal tract was confirmed by microscopic study. The microscope furnishes, we believe, a rather delicate control on the completeness of the removal from the tissues of red blood cells. This is particularly fortunate since in certain tissues, such as skeletal muscle or myocardium, it is impossible to determine in gross just how much of the tissue color is due to residual blood and how much is due to pigments peculiar to the tissues themselves. It is important not to confuse the pigment normally contained within the muscle cells (muscle hemoglobin) with blood pigment. In none of our series was it possible to demonstrate more than a very occasional red blood cell in sections of skeletal muscle. In experiment 339, for instance, even after long-continued search, not a single red blood cell was found. It is hardly necessary to state that normal unperfused skeletal muscle shows considerable numbers of red blood cells. It would seem that the skeletal muscles were particularly easily freed of blood by perfusion. It must be that the conditions of circulation here are such that a free flow of saline to all of the finer ramifications of the blood vessels is quite readily accomplished. Curiously enough the kidney with its double capillary circulation is also left almost completely devoid of blood by perfusion. In contrast, other organs such as the gastro-intestinal tract can be thoroughly perfused only with difficulty. Still in most cases we have been able to remove the blood almost entirely. The spleen and bone marrow are unique in that they possess blood cells which are not in direct communication with the blood stream. A review of the experiments presented shows that red blood cells are made out in fairly large numbers in these tissues.

TABLE 17

Experiment 351. Dog; 9.00 kgm. Hematocrit 41.1 per cent red blood cells

	TOTAL HEMOGLOBIN	TOTAL R. B. C.	BLOOD	PLASMA	PER 100 GRAMS BODY WEIGHT			
					Hemoglobin	R. B. C.	Blood	Plasma
	grams	*cc.*	*cc.*	*cc.*	*grams*	*cc.*	*cc*	*cc.*
Dye method	119.0	381.0	928.0	538.0	1.32	4.23	10.31	5.98
Carbon monoxide method	111.0	358.0	871.0	505.0	1.23	3.98	9.68	5.61
Welcker method								
Total bleedings	95.0	306.0	744.0	432.0	1.06	3.40	8.27	4.80
Extracts								
Heart	0.14	0.45	1.10	0.64	0.0016	0.0050	0.0122	0.0071
Spleen	0.24	0.76	1.86	1.08	0.0027	0.0084	0.021	0.012
Liver	0.61	1.97	4.8	2.8	0.0068	0.022	0.053	0.031
Skeletal muscles	8.3	27.0	65.0	38.0	0.092	0.30	0.72	0.42
Total extracts	9.3	30.0	73.0	43.0	0.103	0.33	0.81	0.47
Total (including extracts)	104.0	336.0	817.0	475.0	1.16	3.73	9.08	5.27

Lungs, liver, kidneys and gastro-intestinal tissue contain only traces of hemoglobin.

Experiment 351. Dog 20–72. Short-haired female mongrel bull terrier. In good condition. Weight, 9.00 kgm. Blood volume determination by dye method at 8:30 a.m.; by the carbon monoxide method at 8:40 a.m. Bleeding and perfusion under ether anesthesia in the usual manner begun at 9:30 a.m. Total duration of perfusion was 2 hours. A total of 18,010 cc. of blood-containing fluid was recovered. The hemoglobin estimations were made in the usual manner.

At autopsy the myocardium is pale. The lungs are quite well perfused except for several small irregular red patches. The spleen is quite small but otherwise appears normal. In areas scattered irregularly over the surface of the liver blood can be seen at the centers of the lobules. On section the liver is pale grey and cloudy. The lobulation is quite indistinct. The stomach shows but the faintest traces of macroscopic blood. The mucosa of the entire small intestine is of a brick-red color. An excess of mucus is present. The large intestine appears to be bloodless. The pancreas is purplish grey and quite edematous. The kidneys and pelvic organs are free of macroscopic blood.

In microscopic sections no red blood cells are seen in the section of myocardium. Sections of lung show a few red blood cells. Blood cells are very numerous in the spleen. The liver is cloudy. Its lobulation is poor. No red blood cells are seen. The villi of the small intestine are quite rich in red blood cells, although but few are to be seen deeper in the mucosa. No signs of blood seen in sections of the large intestine, kidney or skeletal muscle. A moderate number of red blood cells is seen in the pancreas.

The organs and tissues were extracted in the usual manner (see table 17).

In table 18 the quantity of hemoglobin extracted from the various organs is charted. The averages agree fairly well with the findings in the experiments already presented. The amount of hemoglobin is expressed in terms of body weight. It was impossible in this series to express the hemoglobin from each organ in terms of the total weight of that organ on account of the fact that most of the organs were quite edematous from perfusion and it was impossible to gain any very accurate notion of the correct weights of these organs. However, we have noted the weights of the various organs from other dogs killed for other reasons. Assuming that the same figures hold for this series of dogs we may calculate from table 18 that the lungs (normal weight 115 grams) must have contained about 0.01 gram of hemoglobin per 100 grams of tissue. The gastro-intestinal tract (normal weight about 500 grams) being as a rule somewhat less perfectly perfused, contained 0.015 gram per 100 grams of tissue. The liver (average weight about 420 grams) contains about 0.013 gram per 100 grams of liver substance. The spleen which is so rich in residual blood contains about 0.10 gram per 100 grams of tissue or approximately 0.25 gram for the entire organ. This same table shows that the organ extracts have a total of 0.126 gram of hemoglobin per 100 grams of body weight. Of this 0.103 gram or 82 per cent of the total is derived from the skeletal muscles. This seems rather high when it is recalled that microscopic study showed the muscles to contain so few red blood cells. Furthermore, using the results of table 18 as a basis it may be computed that from each 100 grams of skeletal muscle 0.02 gram of hemoglobin was extracted. This is little more than was recovered from a like quantity of lung tissue, but much less than was recovered from the same amount of splenic tissue. Still when it is recalled that the skeletal muscles constitute nearly one-half of the total body weight it will be seen that the total amount of pigment so recovered must of necessity constitute a large part of the total extracts. The relatively large amount of hemoglobin removed from the skeletal muscles by extraction must be derived only to a small extent from blood contained in this tissue. There can be little doubt that the greater part of this pigment is not blood hemoglobin but muscle hemoglobin. The existence within the striped muscle of such a pigment is well known. It has been shown that it is very similar to, if not identical with, blood hemoglobin (11).

From the bones 0.020 gram of hemoglobin per 100 grams of body weight was recovered. Much of this likewise cannot be considered as belonging to the circulating blood. Part of this exists in extravascular

TABLE 18

Summary of hemoglobin values in Welcker experiments

EXPERIMENT	WEIGHT OF DOG	HEMATOCRIT (R. B. C.)	HEMOGLOBIN PER 100 GRAMS OF BODY WEIGHT RECOVERED FROM									
			Total bleedings plus perfusions	Organ extracts								Total bleedings plus perfusions and extracts
				Heart	Lungs	Spleen	Liver	Stomach and intestines	Skeletal muscles	Bones	Total extracts	
	kgm.	*per cent*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*	*grams*
330	4.75	45.3	1.26			0.0044	0.0063	0.0103	0.101	0.033	0.160	1.42
356	8.77	39.8	0.88	0.0024	0.0013	0.0027			0.11	0.025	0.14	1.02
335	8.86	46.2	0.77	0.0015		0.0008	0.0031		0.087	0.005	0.097	0.87
351	9.00	41.1	1.06	0.0016		0.0027	0.0068		0.092		0.103	1.16
333	9.18	53.3	1.24	0.0031		0.0039			0.099	0.027	0.133	1.37
340	10.45	52.1	0.93	0.0015		0.0016			0.146	0.015	0.164	1.09
328	10.46	48.8	1.19		0.0011	0.0021			0.104		0.107	1.30
346	10.68	62.0	1.17	0.0010		0.0014			0.070	0.021	0.093	1.26
342	11.82	48.3	1.18	0.0015		0.0015			0.113	0.015	0.131	1.31
344	13.64	58.9	1.30	0.0014	0.0007	0.0018			0.075	0.018	0.097	1.40
345	13.64	59.5	1.34	0.0013		0.0012			0.094	0.018	0.114	1.45
339	15.45	50.1	1.19	0.0015		0.0017			0.147	0.021	0.171	1.36
349	16.36	46.4	1.17	0.0015		0.0008			0.097	0.029	0.128	1.30
343	18.64	58.0	1.39	0.0006	0.0006	0.0021	0.0018	0.0016	0.102	0.012	0.121	1.51
Average..	11.55	50.7	1.14	0.0016	0.0009	0.0021	0.0045	0.0060	0.103	0.020	0.126	1.27

bone marrow cells which have not yet matured and been thrown into circulation. The spleen contains many red blood cells which are not in active circulation. These cannot be removed by any amount of perfusion. The liver is of a brownish tint due to pigments peculiar to the liver. The presence of these pigments makes it difficult to estimate colorimetrically its blood hemoglobin content unless the latter be fairly large.

In many experiments some of the organs were so well perfused that by extraction such small amounts of hemoglobin were recovered that their quantitative estimation was impossible. In these cases no figures at all are entered in the tables. For this reason the figure for total extracts is slightly smaller than it would be if it had been possible to include the extracts of all organs.

In view of all of these facts obviously considerable difficulty surrounds an attempt to state with any degree of certainty just how much of the total hemoglobin extracted should be considered to be derived from blood cells left behind in the vessels after completion of perfusion. It would seem that the blood hemoglobin must constitute somewhat less than one-half of the total pigment extracted. If, however, we add a small amount for incomplete extraction of the tissues we will probably not err greatly in assuming such a figure. One-half of the total extracts would be 0.063 gram of hemoglobin per 100 grams of body weight. If this amount of hemoglobin be added to that obtained by bleeding and perfusion combined (1.14 grams per 100 grams of body weight) we have a total of 1.20 grams of *blood hemoglobin* per 100 grams of body weight. Even though our assumption that one-half of the pigment extracted is blood hemoglobin is somewhat erroneous, still this figure of 1.20 will be but slightly affected, for a difference of only 5 per cent results, if we neglect the extracts altogether or if we assume the other extreme—viz., that *all* of the extracted pigment is blood hemoglobin.

Muscle hemoglobin and other extravascular pigments of similar nature furnish subject for debate not only in the case of the Welcker method but in the carbon monoxide method as well. The muscle hemoglobin has been shown (11) to be very similar to that of blood hemoglobin and on being placed in contact with carbon monoxide it unites with the latter to form a substance having a spectrum similar to that of the carbon monoxide hemoglobin derived from blood hemoglobin. It seems quite probable that the carbon monoxide, which is administered in the carbon monoxide method and is taken up by the red blood cells, must through processes of diffusion reach and combine to some extent

TABLE 19

Summary of blood volume and hemoglobin values

EXPERIMENT	WEIGHT OF DOG	HEMATOCRIT (R. B. C.)	PER 100 GRAMS BODY WEIGHT														
			Hemoglobin				Red blood cells				Whole blood				Plasma		
			Dye method	CO method	Welcker method		Dye method	CO method	Welcker method		Dye method	CO method	Welcker method		Dye method	CO method	Welcker method
					With extracts	Without extracts			With extracts	Without extracts			With extracts	Without extracts			With extracts
	kgm.	*per cent*	*grams*	*grams*	*grams*	*grams*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
330	4.75	45.3	2.11	1.96	1.42	1.26	5.68	5.28	3.83	3.41	12.53	11.68	8.48	7.52	6.74	6.29	4.55
356	8.77	39.8	1.14	1.03	1.02	0.88	3.64	3.30	3.22	2.78	9.14	8.27	8.12	7.00	5.30	4.80	4.70
335	8.86	46.2	1.24	0.76	0.87	0.77	4.66	2.84	3.26	2.89	10.08	6.15	7.03	6.24	5.27	3.22	3.67
351	9.00	41.1	1.32	1.23	1.16	1.06	4.23	3.98	3.73	3.40	10.31	9.68	9.08	8.27	5.98	5.61	5.27
333	9.18	53.3	1.42	1.67	1.37	1.24	4.79	5.65	4.65	4.19	8.99	10.60	8.71	7.86	4.10	4.84	3.97
340	10.45	52.1	1.51	1.13	1.09	0.93	5.08	3.79	3.67	3.13	9.75	7.27	7.06	6.01	4.58	3.41	3.32
328	10.46	48.8	1.52	1.20	1.30	1.19	5.15	4.07	4.38	4.02	10.55	8.34	8.97	8.22	5.28	4.17	4.48
346	10.68	62.0	1.58	1.42	1.26	1.17	6.16	5.53	4.90	4.54	9.94	8.93	7.90	7.32	3.70	3.32	2.93
312	11.82	48.3	1.79	1.36	1.31	1.18	5.58	4.25	4.09	3.67	11.57	8.79	8.45	7.60	5.76	4.37	4.20
344	13.61	58.9	1.97	1.44	1.40	1.30	6.59	4.82	4.69	4.36	11.19	8.18	7.96	7.41	4.51	3.30	3.20
345	13.61	59.5	1.69	1.35	1.45	1.34	5.78	4.62	4.98	4.59	9.72	7.76	8.34	7.68	3.85	3.07	3.31
339	15.45	50.1	1.79	1.44	1.36	1.19	5.55	4.47	4.22	3.68	11.07	8.92	8.40	7.33	5.42	4.37	4.11
319	16.36	46.4	1.33	1.28	1.30	1.17	5.02	4.46	4.51	4.06	10.83	9.60	9.71	8.74	5.69	5.05	5.12
343	18.64	58.0	2.08	1.51	1.51	1.39	6.01	4.37	4.41	4.05	10.42	7.53	7.60	6.98	4.29	3.10	3.13
Average	11.55	50.7	1.61	1.34	1.27	1.15	5.28	4.39	4.18	3.77	10.39	8.69	8.27	7.44	5.03	4.21	4.00

with the muscle hemoglobin. It seems more than likely that the carbon monoxide method indicates not only the blood hemoglobin but takes in the muscle hemoglobin as well. We know of no other compound in the mammalian body which possesses an especial affinity for carbon monoxide. By physical solution at the carbon monoxide tension obtaining in the plasma of blood one-half saturated with carbon monoxide not more than 2 or 3 cc. of this gas could be taken up by all of the body fluids, even assuming the latter to constitute a very great proportion of the body. The error arising from this source would not exceed 2 or 3 per cent. It is improbable that this amount of CO could be held in body fluids in the presence of unsaturated hemoglobin.

Further study of table 19 shows in general the same figures for hemoglobin and red cells by the CO method and the Welcker method. The average figures show that the CO method gives figures which are 5 per cent greater than those recorded by the Welcker method for hemoglobin and tissue extracts combined. In general the figures correspond within reasonable limits. It is more simple to explain the occasional higher Welcker figures because we can assume incomplete equilibrium and mixing of the circulating CO. When the CO figures are higher than the Welcker figures we must assume some limitations in the accuracy of the technical procedure. The first experiment in table 19 (no. 330) is the only one which shows a startling discrepancy between the CO and Welcker figures. We have observed in other experiments that small or young dogs apparently had an unusual capacity of removal of CO from the blood stream. This point is being investigated further. Other than this the probability of a technical error must be considered as the figure of 5.28 cc. per 100 grams body weight is much above the average of 4.39. We see, however, that individual variations in this group of dogs is quite considerable—5.65 cc. per 100 grams, a maximum and 2.84 cc. per 100 grams, a minimum—average of 4.39 cc. This shows 100 per cent fluctuation between minimum and maximum. We believe that the accumulation of more data will show that the larger dogs show less variation in blood volume factors. These variations are not associated with anemia as shown by the hematocrit column. The plasma volume figures show less notable fluctuations—a maximum of 6.74 cc. per 100 grams body weight—a minimum of 3.70 cc. and an average of 5.03 cc.

From table 19 it is seen that 1.34 grams of hemoglobin per 100 grams of body weight are found by the carbon monoxide method. This is considerably higher than the amount which could be recovered in the

Welcker method by bleeding and perfusion combined. If, however, the total extracts are included in the Welcker determination we have a figure which approximates the value of 1.34 obtained by the carbon monoxide method. If experimental errors be taken into account these two values may be considered as almost identical. That this total hemoglobin (blood plus muscle hemoglobin) of the Welcker method should approximate the total hemoglobin, as determined by the carbon monoxide method, is evidence in favor of the view just outlined that by the carbon monoxide method the muscle hemoglobin as well as the blood hemoglobin is estimated.

DISCUSSION

Before attempting further to compare and correlate these three blood volume methods let us emphasize what has already been stated, viz., that the values *directly* obtained by each of the methods are in all cases tabulated in bold type. These figures include for the dye method the plasma volume; for the carbon monoxide and Welcker methods, the figures for hemoglobin and red blood cell volume. All other figures are derived *indirectly* from these figures on the *assumption* that the red blood cell volume bears a ratio to the plasma volume identical to the ratio of reds to plasma observed in a centrifugalized sample of blood drawn from the jugular vein. In this assumption is involved the idea that the plasma and red blood cells are thoroughly and uniformly mixed within the vascular system and that a sample of blood taken from the jugular vein contains neither more nor less than its proper proportion of red blood cells. But this assumption falls to the ground if it can be shown that conditions in certain parts of the vascular system (e. g., certain of the smaller vessels) are such as to favor undue retention of either plasma or red blood cells. We shall discuss presently certain evidence in favor of the view that the blood is far from being uniformly mixed. At any rate while the assumption mentioned above is under suspicion it is well to trust only the figures placed in bold type. Most previous investigators have unreservedly accepted the other figures also. These figures are included in our charts so that our results may be conveniently compared with and correlated with the results of previous workers.

We believe that the evidence already presented entitles one to conclude that the carbon monoxide method or the Welcker method furnished fairly reliable data concerning the total red blood cell volume. As has been noted above the maximum figures by either method in all probability represent a figure which is somewhat greater than the true red blood cell volume. For in the Welcker method a part of the pigment extracted is not blood hemoglobin, but muscle hemoglobin. This muscle hemoglobin probably unites with carbon monoxide and thereby causes the carbon monoxide method to give values that are equally too high. If we deduct from the results of both methods an amount equal to one-half of the total organ extracts we shall have a red cell volume of 4.18 cc. per 100 grams of body weight by the carbon monoxide method and 3.97 by the Welcker method (see table 19). At any rate in round numbers 4.1 cc. per 100 grams must represent a very close approximation to the true red blood cell volume.

It must be emphasized repeatedly that the volume of *actively circulating red cells* is definitely less than the total *body hemoglobin* which, of course, includes muscle hemoglobin (Myohematin) and red cells in stagnant sinusoids in spleen and red marrow. It appears probable that this *immobile hemoglobin* represents 5 to 10 per cent of the total body hemoglobin which is concerned in the dilution of inhaled carbon monoxide. The CO method does not permit us to differentiate between the rapidly circulating hemoglobin and the immobile or fixed hemoglobin. Perhaps some method may be devised by which this difficulty can be surmounted.

The proof as to the size of the *plasma volume* is somewhat more difficult to obtain. To summarize, there are many methods for the determination of plasma volume. In all of them the principle is the same as the principle of the dye method. Dyes other than vital red have been used. Acacia, gelatin, dextrose, antitoxin and hemoglobin may also be substituted for the dye. All of these methods agree in indicating that the plasma volume is of the magnitude stated (4.8 cc. per 100 grams of body weight). It is difficult to believe that all of the substances thus used for the determination of plasma volume would be eliminated to any great extent in the 4-minute period allowed for their admixture with the plasma. Especially difficult is it to believe that substances so diverse in nature would all be so much more rapidly eliminated in the first few seconds than thereafter. The fact that dye once introduced can be recovered in large measure by perfusion indicates that destruction or elimination of dye is not great. Furthermore, the elimination of dye from circulation may be studied by withdrawing samples of blood for analysis at varying intervals of time following injection of the dye. A curve may be plotted to indicate these values. If this curve be drawn backward to cover the period prior to the taking of the 4-minute sample the result indicates that not more than 5 per cent of the total dye injected is eliminated within the first 4 minutes. If a correction be made for this, the method shows that there are 4.8 cc. of plasma per 100 grams of body weight. We hope very soon to present experimental data to supplement this brief review of the plasma volume factors.

By *blood volume* is meant the sum *total of plasma volume, total red blood cell volume* and *white blood cell volume.* We have just discussed a method which determines plasma volume (dye method) and two others (carbon monoxide, Welcker) which determine red blood cell volume. No thoroughly reliable data concerning the total volume of white blood cells exists. Our hematocrit figures indicate that on an average the white blood cells constitute about 1.3 per cent by volume of the circulating blood. The work of Brodin, Richet and Saint-Girons (12) indicates that only about 50 per cent of all the white blood cells are in active circulation, the others being retained presumably in some of the smaller vessels. On the basis of these observations the white blood cell volume must constitute about 2.3 per cent of the total blood. If so, there are approximately 0.21 cc. of white blood cells per 100 grams of body weight. In this case the blood volume would equal per 100 grams of body weight, 4.8 cc. of plasma plus 4.2 cc. of red blood cells plus 0.2 cc. of white blood cells—a total of 9.2 cc. This we feel represents fairly accurately the total blood volume in our series of dogs. We wish to emphaszie our belief that it is only by a combination of methods that a proper estimate of the blood volume can be obtained. There is no justification for the custom of assuming that the plasma volume and red blood cell volume bear to each other the same ratio as is found in a centrifugalized sample of blood drawn from any of the usual sources. Yet this is exactly what is done when by the use of the hematocrit the total red blood cell volume is derived on the basis of a plasma volume previously determined by the dye method or the acacia method or any of the other plasma volume methods. It is equally incorrect to attempt to estimate the plasma volume when merely the red blood cell volume has been determined by either the carbon monoxide method or the method of Welcker. Such estimations involve the assumption that the ratio of red blood cells to plasma in blood drawn from the jugular vein is a ratio which is representative of the blood as a whole. A great amount of work has been reported which indicates that the distribution of blood cells in the body is a very changeable factor. The well-known observations of Cohnstein and Zuntz (13) may be cited in this regard. By microscopic study of living preparations they found that the smaller vessels became crowded with red blood cells at the expense of plasma under certain conditions of vascular relaxation. Under conditions of vasoconstriction the red blood cells became less numerous and these fine vessels contained relatively more plasma. Much of the work on red blood cell count changes with high altitude and with exercise and the like may be explained on the basis of altered distribution of the blood cells. Confirmation of such theories is lacking, for as yet no investigator has studied these problems with a combination of a plasma volume method and a red blood cell method. The mere fact that while the red blood cell count rises, the red blood cell volume (or the total oxygen capacity of the blood) is unaltered does not indicate what changes may be occurring in the total volume of plasma. The work of Lamson shows that under certain conditions the blood cells may tend to accumulate in the liver (14).

We believe that the results of the present paper indicate in a way hitherto unsuspected, the variable admixture of the plasma and red blood cells. A review of table 19 shows that on an average there are 5.03 cc. of plasma per 100 grams of body weight. As has been already explained it seems probable that about 4 or 5 per cent of the dye injected is eliminated in the 4 minutes allowed for mixing of the dye with the plasma. Granting that this is true the corrected plasma volume is about 4.8 cc. of plasma per 100 grams of body weight. According to the same table there are about 4.2 cc. of red blood cells per 100 grams of body weight. The plasma together with the red blood cells equals the sum of these two figures or 9.0 cc. per 100 grams of body weight. If to this the probable white blood cell volume (0.2 cc. per 100 grams of body weight) be added, a sum-total of 9.2 cc. per 100 grams of body weight is obtained. This represents, we believe, a close approximation of the total blood volume. Since the red blood cells constitute 4.2 cc. of this 9.2 cc. it is easily seen that the red blood cells constitute 45.5 per cent of the total blood. This value represents the true red blood cell hematocrit. If, for instance, all of the plasma and blood cells could be removed from the body, placed in a large container and completely mixed, a sample of this blood on centrifugalization would be found to contain 45.5 per cent of red blood cells. In the living animal it is impossible to secure any such ideal sample, since it is impossible to take blood from all parts of the vascular system at once. A sample of blood taken from the jugular vein contains 50.7 per cent of red blood cells (table 19)—a figure considerably higher than the true 45.5 per cent. It would seem then that blood taken from the jugular vein is considerably too rich in red blood cells. In an effort to locate other regions having a corresponding poverty in red blood cells we have drawn samples from the femoral vein, carotid artery, femoral artery, portal vein and other large vessels. Needless to say obstruction to the flow during the taking of all such samples was carefully avoided. It is not necessary to present all of the figures in detail. It will suffice to say that blood drawn from all of these sources was found within very narrow limits to contain the same percentage of red blood cells as was found in blood drawn from the jugular vein. We next turned our attention to the smaller vessels. It is very difficult to obtain any very exact information regarding the richness in red blood cells of the blood contained in such vessels. However, certain microscopic findings enable us to draw some general conclusions. Almost as soon as the microscopic circulation of the blood was first discovered it was observed that the flow in the smaller vessels was not at a uniform rate in all parts of the cross section. It was noted that in the central or axial portion of the stream the flow is much more rapid than in the peripheral portion of the stream. Furthermore the red blood cells are to be seen almost exclusively in the central rapidly moving part of the stream. If the laws governing the flow of simple liquids in tubes hold here, the center of the stream moves at a rate equal to twice the average velocity of the stream. The "still space" represents a body of plasma which is relatively stagnant—withdrawn from the circulation, as it were. The width of this layer undoubtedly varies in different vessels and under different conditions of flow. It is ordinarily stated that this layer has a width of at least one-eighth of the diameter of the vessel. If this be so it can easily be shown that this "still space" constitutes about 44 per cent of the cross sectional area of the vessel. Of course the plasma in this "still space" is not perfectly stationary and hence is not completely withdrawn from circulation. The rate of movement in any part of the vessel is approximately inversely proportional to its distance from the center. At this rate the plasma in the inner fourth of this so-called "still space" may be thought of as moving at the same rate as the innermost or "axial core" while the outer three-fourths of the "still space (i. e., the three-fourths nearest the vessel wall) may then be considered as being perfectly stationary. If, then, the "still space" constitutes 44 per cent of the cross sectional area of the vessel and three-fourths of this "still space" can be considered as being stationary it follows that 33 per cent (75 per cent of 44 per cent) of the contents of the vessel is stagnant plasma. The other 67 per cent of the content of these small vessels is the actively circulating blood of the body and in our series of animals contained 50.7 per cent by volume of red blood cells. On an average there were 4.1 cc. of red blood cells per 100 grams of body weight. If these cells were all actively circulating our hematocrit would indicate that they should be accompanied by 4.0 cc. per 100 grams of body weight of actively circulating plasma. Our experiments show that the sum total of plasma is about 4.8 cc. per 100 grams of body weight. The difference between 4.8 and 4.0 is 0.8—a figure which represents the number of cubic centimeters of stagnant plasma per 100 grams of body weight. If the true blood volume be 9.0 cc. per 100 grams of body weight it is seen that the stagnant plasma constitutes about 9 per cent by volume of the total blood. All of this plasma could be stored away in the "still spaces" of the smaller vessels on the assumption that such small vessels contained one-third of the total blood. For, as was estimated above, about 33 per cent of the content of the smaller vessels consists of stagnant plasma for which we must account. It does not seem unreasonable to believe that one-third of the total blood is contained in the small vessels possessing an axial stream, particularly when it is recalled that the total cross section of the finer arterioles and venules has been estimated to be several hundred times the cross section of the aorta. Furthermore, the length of some of the arterioles is considerable. The total combined content of all such vessels must be very great.

Unfortunately previous microscopic studies and measurements do not enable us to establish conclusively our theory that the total volume of the "still space" is great enough to account for all of the plasma which we have shown is stored somewhere. We hope within the near future to be able to collect definite first-hand data on this point. These considerations do, however, establish the fact that there is within the "still spaces" at least a considerable quantity of stagnant plasma.

We must not overlook the possibility that plasma may also be "stored" in other regions. Thus Cohnstein and Zuntz (13) held that there are capillaries which are filled with plasma to the exclusion of red blood cells. Some investigators may wish to maintain that a certain part of the extravascular lymph spaces are quite accessible to the dye and that the lymph constitutes some of the stagnant part of our "plasma volume." The evidence now at hand is somewhat against this view. Our theory involving the "still spaces" seems to cover all known facts both from a qualitative and quantitative standpoint. This view in no way conflicts with the work of those who wish to maintain that under certain conditions of circulation, red blood cells may be stored or deposited to excess in certain parts of the vascular tree. Nor do we wish to deny that under certain conditions the plasma volume may vary to a considerable degree. Further evidence is being collected on these points.

We wish to emphasize the view already presented to explain why the total "blood volume" as obtained by the dye method constitutes 9.5 to 10.5 cc. per 100 grams of body weight, while the total "blood volume" obtained by the carbon monoxide or Welcker method constitutes but 8 cc. per 100 grams of body weight. For blood drawn from the jugular vein is composed of approximately equal parts of plasma and blood cells. Anyone who did not know of the plasma stored away in the smaller vessels might make the mistake of thinking that the blood volume was twice the plasma volume or twice the total blood cell volume. In case he had already established by the dye method the fact that there are 4.8 cc. of plasma per 100 grams of body weight, he would be led to conclude there there is twice this amount of blood i. e., 9.6 cc. of blood per 100 grams of body weight. On the other hand, if he had established by the carbon monoxide or Welcker method that the red blood cell volume is 4.1 cc. per 100 grams of body weight he would conclude that there are but 8.2 cc. of blood per 100 grams. As our discussion shows, such calculations are not based upon sound premises. These workers should combine the plasma volume obtained by the dye method with the red blood cell volume obtained by the carbon monoxide or Welcker method. The result would be 8.9 cc. per 100 grams of body weight. If desired, one might add 0.2 cc. for white blood cells. In this case the observer would conclude that there are 9.1 cc. of blood per 100 grams of body weight. This, we believe, represents a correct approximation of the actual blood volume.

SUMMARY

Three standard blood volume methods are compared by the same workers under uniform conditions with a carefully controlled technic. The dye method, the carbon monoxide method and a modified Welcker method are all used on the same dog within a short space of time. The comparative value of these figures is thereby enhanced.

The dye method determines with reasonable accuracy the plasma volume but not the red cell volume. The carbon monoxide or Welcker method determines the total hemoglobin or red cell volume but not the plasma volume.

The dye method in normal dogs gives a plasma volume of about 4.8 cc. of plasma per 100 grams body weight. The carbon monoxide method in normal dogs gives the red cell volume as 4.2 cc. per 100 grams body weight. There are probably about 0.2 cc. of white blood cells per 100 grams body weight. In our opinion the *correct blood volume* is the sum of these figures or 4.8 cc. plasma + 4.2 cc. red cells + 0.2 cc. white blood cells = *9.2 cc. blood volume* per 100 grams body weight.

We believe other investigators have fallen into error due to *calculation of the total blood volume* based on the red cell hematocrit—usually 50 per cent in normal dogs. This gives erroneous figures of about 10 cc. per 100 grams body weight for the dye method and about 8 cc. per 100 grams body weight for the carbon monoxide method.

This error comes from the assumption that the blood cells and plasma are uniformly mixed in all parts of the circulating system. There is sufficient evidence to point to an *excess of plasma* over red cells in arterioles and capillaries. The axial cell stream in arterioles, for example, gives a marginal "still space" in which a considerable amount of plasma may be stored.

The absolute value of circulating red cells must always be somewhat smaller than the carbon monoxide figures which express the total *body hemoglobin,* inclusive of muscle hemoglobin and hemoglobin in red marrow and spleen sinusoids. The amount of this *immobile body hemoglobin* is about 5 to 10 per cent of the total body hemoglobin.

The true *total blood volume* can be obtained by the use of the carbon monoxide method (red cell volume) plus the dye or similar method (plasma volume). The sum of these figures gives the true blood volume.

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PERISTALSIS IN HEALTH AND DISEASE

First Caldwell Lecture

VIII

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PERISTALSIS IN HEALTH AND DISEASE[*][†]

First Caldwell Lecture

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MR. PRESIDENT, Members of THE AMERICAN ROENTGEN RAY SOCIETY: I wish first to thank you for the great honor you have done me in asking me to give the first Caldwell Lecture. It is a pleasure to join with you in remembering the name of one of the martyrs of medicine—a man who gave his life for the advancement of science. He was a pioneer, an inventor, a research worker, a versatile, enthusiastic and lovable man; and we are all losers by his death. We shall honor him best by carrying on the work which he loved so much, and which meant everything in life to him. I believe we shall carry it on most rapidly when we do as he did, when we venture out into "pure" science—into physics, chemistry and engineering, to improve our apparatus; and into biology, physiology and bio-chemistry to explain our findings in the body. How much more rapidly our transformers and tubes would have been improved if there had been more men like Caldwell at work in the field; and how glad we should be to-day that some manufacturers have at last seen the incalculable benefits that can come through proper co-operation with a physicist like Dr. Coolidge!

Every advance in technique brings its problems of interpretation. I shall never forget my delight when, in 1912, I got my first radioscope from Vienna and saw the peristaltic waves coursing over the stomach. At first it seemed as if all my desires had been fulfilled, but in a few months this feeling of satisfaction gave way to one of worry over the many things which I saw but could not interpret. I went East and told my troubles to Cannon, who frankly admitted that many of our pathologic findings are not explainable on the basis of the myenteric reflex or the acid control of the pylorus. He gave me room in the laboratory and told me to go to work on these problems myself. He showed me how to open an (anesthetized) animal's abdomen under salt solution so that I could watch the peristaltic movements. Two or three days of this and I saw that the problems I had set for myself were too big and too complicated. I was like a man who would repair a wireless telephone without first knowing the structure and inner workings of the constituent batteries, induction coils, condensers and magnets. It was clear that I had to begin back near the beginning, on prob-

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lems which to an onlooker might appear to have no promise of practical value. The intestine is a tube made up of smooth muscle and it seemed to me that if I could learn more about the properties and peculiarities of that tissue I would come near to solving some of the riddles of peristalsis. Hence it is that for the last seven years I have turned somewhat aside from the clinical journals and have delved into the literature of the experimental zoologists, the physiologists and the comparative anatomists. I have studied smooth muscle in organs other than the bowel—in the bladder, the ureter, the vas deferens, the uterus, the arteries and the iris. Many helpful suggestions have been gotten from studies on snails, sea-cucumbers, anemones, jelly fishes, clams, worms and crayfishes. I have found the medical indexes almost useless in this work, because the most helpful articles are often concealed behind the most uninviting titles. Thus, who would have expected to find an explanation for the gastric upsets of fevers and asthenic states in an article on the swimming plates of a tiny water organism?—yet there I believe it is. The author is Professor Child, a zoologist, and the article is entitled, "The Gradient in Susceptibility to Cyanides in the Meridional Conducting Path of the Ctenophore Mnemiopsis." [1]

It is unfortunate that we practicing physicians must often miss the most thought-stimulating articles because they are buried away in the highly technical journals which we do not ordinarily read. Some day we may have a better abstracting service, but in the meantime we shall probably have to do occasionally as the Israelites did when they approached the Promised Land; we shall have to send out spies to look over this field which we ourselves have so little time to explore, and to bring back some of the fruits. When asked to speak before you to-day, it seemed to me that the best service I could render would be to bring to you some of the ideas which I, a practicing physician and radiographer, have gotten during my sojourn among the physiologists.

THE AUTONOMY OF MANY PARTS OF THE BODY

The first of these ideas is that *the forces which bring about, modify and control peristalsis must be looked for mainly within the walls of the gut itself.* I cannot too strongly emphasize this point, because it seems to me that the failure to grasp it is the greatest stumbling block to further advance in the knowledge of our subject. Whenever I describe some of the regional differences in behavior which can easily be demonstrated in the excised stomach and bowel, someone is almost sure to say: "Oh, that is due simply to the autonomic and the sympathetic." Another settles the whole problem by calling it a "Reflex." When I point out that the peculiarities persist in little pieces of muscle which have been cut out and kept in the ice box for several days, these individuals still ascribe everything to the activities of the canny little ganglion cells in Auerbach's plexus.

Now, the most paralyzing thing in scientific work is a facile explanation which puts a stop to further curiosity without really advancing our knowledge of the subject; and I have never been able to see the value of pushing the explanation for a mechanical phenomenon out of the organ in which it might be studied, and into a tiny ganglion where we can hardly follow it. It seems to me that many even of the teachers of physiology have a wrong idea of the nervous system and its relation to the viscera. They look at it somewhat as an electrical power house which not only supplies the motive force but controls the activities of the various trains running over a railroad. My analysis of the literature makes me feel that we should look at it more as a telephone switchboard with wires which carry nothing but messages of warning and advice from one engineer to another. The trains supply their own power; and the differences in speed and other activities are due to peculiarities in fuel, differences in the gradient of the road, etc.

Anyone who will study the behavior of the lower forms of life before and after removal of their nervous systems will see that the nerves are there primarily to expedite conduction. The ganglionic cells are nutritional centers and not storehouses of wisdom and power. Some of the most complicated "reflexes" are performed by decerebrate animals or by excised parts of these animals. Loeb, Parker and others have shown clearly that the mechanisms are local and comparatively simple.

THE MYOGENIC NATURE OF THE RHYTHMIC CONTRACTIONS

Similarly, in the gastro-intestinal tract we find that after the preliminary shock has worn off, digestion goes on quite normally after complete section of the vagi and the splanchnics. We get good peristalsis in the stomach and intestine that have been removed from the body and either perfused or placed in oxygenated Locke's solution. We get active peristalsis in small segments of gut and in pieces of muscle stripped from the wall. It is clear then that the gastro-intestinal tract is autonomous: it carries within itself all the mechanisms essential to peristalsis.

The next question is, what part is played by the muscle and what part by Auerbach's plexus? Practically all writers on the subject state that the rhythmic contractions are due to stimuli coming from the ganglion cells of the plexus. They base this opinion on the first few papers by Magnus, who found that strips of circular muscle from which the plexus had been removed did not contract rhythmically. Unfortunately no one seems to have noticed that in his fifth paper,[2] Magnus admits that these same plexus-free strips did beat when he added a little physostigmin, strophanthin or barium to the solution. Moreover, no one notices the work of Gunn and Underhill[3] who repeated these experiments, and by taking greater precautions to avoid trauma, obtained plexus-free strips which would contract rhythmically without the help of tonic drugs. This is what we should expect, because it has been well proven in many ways that rhythmic contraction is a function of muscle itself.

It is clear then that the man who would understand peristalsis and the various activities of the stomach and bowel must pay particular attention to the properties and reactions of the gastro-intestinal muscle. Some may ask: what then is Auerbach's plexus good for? It almost undoubtedly serves for the conduction of stimuli and the coordination of movements; it enables the muscle to respond properly to stimuli coming from the underlying mucous membrane, and it helps in some way to keep that muscle from contracting down into a hard knot. Such contractions are well known to biologists, and somewhat resemble the spastic paralyses of striated muscles.

THE AUTONOMIC AND THE SYMPATHETIC

The next question is: what is the significance of the vagus and sympathetic nerves running to the intestine? The average clinical writer to-day states that the autonomic (vagi and sacral nerves) stimulate, and the sympathetic fibers inhibit the intestine. Disease is supposed to follow an unbalance between these two effects. This unbalance can be diagnosed and corrected by the use of certain drugs which are supposed to be elective in their actions. These theories of vagotonia and sympathicotonia have had a strange fascination for the medical mind; they have been dragged in with the utmost assurance to explain all sorts of disease states; and I believe have had a most unfortunate influence on our conceptions of gastro-intestinal physiology. It may be that these theories will eventually prove useful; but in my opinion their foundations are so shaky that some day the whole edifice is going to go. In the following brief discussion I can only point out a few places in which the proponents of these theories have made things much simpler than they really are.

Although in the main, the vagus tends to stimulate and the sympathetic to inhibit the stomach and bowel, these effects are generally transient, often indecisive and not infrequently reversed. They vary with the strength of the stimulus and with the condition of the muscle. The next objection is that the whole theory of "nerve endings" and "intermediate substances" is shaky. Leading pharmacologists have for some time been calling attention to the many contradictions in the literature of the subject, and have warned us to be careful in accepting physiologic conclusions based upon the supposed activities of the various nerve poisons.[4]

Perhaps the strongest objection to the recently revived theories in regard to the autonomic and sympathetic systems is that they make it appear that the sympathetic nerves with the celiac ganglia constitute a separate and distinct brain system which can be antagonistic to, or out of harmony with the central nervous system. This view is entirely at variance with the facts, which have been collected and discussed in a masterly way by Gaskell in his monograph on "The Involuntary Nervous System."[5] He shows that the involuntary nerves and ganglia are a part of the central nervous system; they are connected with it just as the voluntary nerves are, and they have developed from the same embryonic cells. The main difference is simply that the motor ganglia which in the voluntary system are found in the anterior horns of the cord, have migrated, some as far as the paravertebral ganglionic chain; some into the solar plexus, and some into the nerve nets in the walls of the hollow organs. Hence it is that the rami communicantes or preganglionic fibers in the sympathetic system, and most of the efferent fibers in the vagus are simply elongated connector neurones such as we find between the motor and sensory roots in the cord, and in the pyramidal tracts. Furthermore, it has been shown that there are no commissural fibers between the different sets of sympathetic ganglia such as would have to be present if these ganglia were to mediate reflexes like an abdominal brain.

VALUE OF THE EXTRINSIC NERVES

We learn then from Gaskell the same lesson that we have had from Loeb and from Parker: that the nerves are there to conduct, and not to exercise faculties requiring almost human intelligence. There are times when the animal as a whole needs to communicate with its digestive tract; there are times also when the tract must communicate with the body. There are many times when one end of the tract must communicate with the other; and on all these occasions the extrinsic nerves come into play. The vagi carry feelings of hunger and of satiety from the stomach to the brain; they help in adjusting the tone of the stomach wall to the food coming down the esophagus; and they carry the stimuli that give rise to the psychic secretion of gastric juice. If the food must be rejected by vomiting they carry the impulses which bring the abdominal muscles to the aid of the stomach. They probably carry messages from the digesting tract which make the animal feel comfortable and sleepy. The splanchnics serve largely to quiet the tract and to stop digestion when the body is distressed or injured. The extrinsic nerves probably have much to do with the digestive upsets with disease elsewhere in the body; but we shall see later that these changes can be accounted for also by actual damage to the gastro-intestinal muscle.[6]

I regret that I have had to spend so much time on this subject of nervous control; but it seems that the human mind is not so ready to look for new explanations for well-known phenomena, or even to accept them when found, until its contentment with the old explanations has been disturbed. Now that we see how autonomous the tract is and how dangerous it is to rely on theories of ganglionic control, we should be the more eager to learn all we can about the gastro-intestinal muscle.

SMOOTH MUSCLE

As you know, smooth muscle is made up of spindle-shaped cells which vary in size, shape, number of nuclei, etc., in different animals and in different parts of the same animal. As a rule it contracts more sluggishly than striated muscle does; it takes longer to get started, and it is slower in recovering its original length. After a number of strong stimuli or sometimes after only one, it may become quite refractory. After a long rest it may seem to get on a hair trigger again so that it responds powerfully and explosively to a slight stimulus. That is the condition of the digestive tract after the night's rest; and it probably has much to do with the fact that most of us have the daily bowel movement in the morning, immediately after breakfast. With an animal open under salt solution, one can often start a rush wave down the bowel by pinching the duodenum. For some time afterwards, similar pinches will have no effect, but if we wait long enough we will again find the bowel so sensitive that the slightest stimulus will start a wave.

Another characteristic of smooth muscle is its ability to maintain a firm and lasting contraction without fatigue. We see this in the muscles which close the shells of bivalves and we see it in the wall of the colon. It is interesting that the muscle in a bivalve consists of two parts: one which closes the shell and the other which locks it closed. By cutting first one and then the other it can be shown that neither one can do the work of the other. Similarly, if one of you will try to hold his arm out perpendicularly to his body he will soon find it a most painful and fatiguing experiment. The deltoid was not designed for such heavy work, but the glutei and back muscles are carrying much heavier loads all day, and they do not complain. There are all kinds of muscles, all suited to different purposes. Some, like those in the wings of insects, must contract 300 times a second; others like those in the wings of a hen have little to do. Those who think all muscle is the same forget the diffenences between the white and dark meats of chicken, between the heart and the gizzard; between the tenderloin, roundsteak and tongue. I have gone into these differences so at length to prepare you for the thought that there are big differences between the muscle in the cardiac and pyloric ends of the stomach;[7] between that in the small intestine and that in the cecum [8] and colon.[9] The muscle on the lesser curvature near the cardia is soft to the touch like coagulated fibrin; that in the pyloric antrum is tough like gizzard and has a different color. Stimulate the two with an electric current or with a pinch and you get two entirely different contraction curves; put them into warm oxygenated Locke's solution and you get two different types of rhythmic activity. These differences were to be expected when we remember that the upper and lower ends of the stomach have different kinds of work to do. The upper end serves largely as a hopper to hold the food; the lower is the mill that does the heavy work. More of these local peculiarities will be described later.

Another characteristic of smooth muscle in hollow organs is its responsiveness to tension. Most of the manifestations of peristalsis are brought about and regulated largely by the internal pressure due to the presence of food or gas. Cannon has shown that the rhythmic segmentation in the small intestine is due simply to the fact that those muscle fibers which are stretched tend to contract.

Smooth muscle shortens also under the influence of direct irritation. Thus we find contraction of the cardia, pylorus, ileo-cecal sphincter and anus when there is ulceration or inflammation near by. We find hour-glass contractions of the stomach opposite ulcers on the lesser curvature; and shrunken and irritable duodenal caps with ulcers in that region.

PERISTALSIS

If we stimulate the smooth muscle in a tubular organ like the intestine or ureter, we get a contraction which produces a tonic ring. From this ring, waves are given off in both directions. They remind one of the ripples which arise at a point where a stone has been thrown into a pond. The impulse spreads from muscle fiber to muscle fiber, and need not be mediated by nerves, ganglia, centers or reflexes. I have observed, after electrical stimulation, similar waves spreading both ways along the segments of a recently voided tapeworm; and I have seen them traveling away from the ridge which forms when one strikes the irritable pectoral muscles of a consumptive. It seems to me that the stimulus probably increases the chemical activity at the point where the tonus ring forms; it raises the metabolic rate, and stimuli spread out on both sides down gradients of chemical activity.

GRADIENTS

Returning to the simile of waves spreading in water, it seems to me that some tubular organs may be likened to ponds which are level to begin with; others are more like rivers which have definitely established gradients. In the first case the waves spread equally well in all directions; in the second, the waves spread better down stream than up. Perhaps I can illustrate my point best by showing the evolution of the fixed gradient in the heart. As you know, in that organ the beat follows a gradient of rhythmicity from the sinus to the ventricle. It was Gaskell who showed that if we cut a heart into three or four pieces the one containing the mouths of the great veins will show the greatest tendency to beat rhythmically, and will have the fastest rate. The ventricle will be slow to start beating and will have a slow rate. Now, when we turn to the primitive heart of the sea slug (aplysia), we find a tube which apparently has no constant gradient in either direction. Its beat arises now on one side and now on the other, depending on where the blood produces the greatest tension. Hunter could find no sign of a gradient in the heart of one of the ascidians. In these animals the beat runs for a while towards the viscera and then for a while towards the gills. The pace-making end seems to get fatigued; its rate is slowed and finally the other end is able to assume the pace for awhile. A constant direction of contraction may be maintained by electrical stimulation of either end of such a heart. It seems to me that Hecht's [10] studies on ascidia atra show us the very beginning of the fixed gradient which we find in the hearts of the higher animals. He found that although the heart of the ascidian reverses its beat from time to time, the sum of the advisceral beats is about twice that of the abvisceral. Moreover, as we should expect if the gradient is a little better in the advisceral direction, the rate of conduction is definitely faster in that direction than in the other. If a wave is started in the middle of the heart going both ways it tends to efface the abvisceral waves which according to our theory would have the smaller momentum. It is interesting also that under slightly adverse conditions, as after warming the water or after diluting or concentrating it, it is the abvisceral beat which is suppressed. We find a little more stable, but still reversible heartbeat in the sharks and rays. In them the slightest stimulus to the bulbus aortae will reverse the beat and the same stimulus to the sinus will restore it. Even in the higher vertebrates the heartbeat can be reversed temporarily by agencies which lower the rhythmicity of the auricle or raise that of the ventricle.

There is considerable evidence now that peristalsis in the ureter follows a gradient of rhythmicity from the kidney to the bladder.[11] and I have been collecting data which suggest that there are similar gradients in the vas deferens and the fallopian tubes. We may perhaps be able later to state it as a law that the direction of transport of material in a tubular organ depends on gradients of rhythmicity, tone, irritability and metabolism. When we come to think of it, our experience with engineering and mechanics should have led us long ago to look for gradients in these muscular tubes. We know that water in a pipe follows gradients of gravity or of pumping pressure; electricity flows along gradients of voltage, the winds follow gradients of barometric pressure, etc.

GRADIENT IN THE INTESTINE

Now what evidence have we that there is a gradient in the gastro-intestinal tract? As far as rhythmicity goes the evidence is overwhelming. It is a simple thing to open an animal (rabbit) under salt solution and to demonstrate that the rate of rhythmic contraction varies from about 20 per minute in the duodenum to 10 per minute in the lower ileum. It is easy also to cut out short segments of the bowel and to show that their rate of rhythmic contraction continues to vary inversely as the distance from the pylorus. A similar gradient can be shown in strips of muscle excised from the wall of the stomach. The fastest rate is found in the strip from the lesser curvature near the cardia. It is harder to show the gradient in the colon, but that was to be expected. Remember that the large bowel is more sluggish than the small; it lets the contents lie in one place for long periods of time and waves can go in either direction over the cecum and ascending colon. Hence it is that the excised muscle is slow to start beating, its rate is slow; it tends to contract down into a hard knot and stay that way; and the gradient is poor and often reversed. In the small intestine of the rabbit and white rat the rhythmic gradient is so fixed, and so intimately "built into" the structure of the intestine that one can determine the oral and aboral ends of short excised segments by counting the rates at the two ends. It has been shown also that when sections of small intestine are cut, turned end for end and anastomosed again, they will transport liquids but not solids, because the original direction of peristalsis is maintained as long as the animal lives. This experiment shows that the gradient is basic and not the result of functional adaptation. Further evidence for that conclusion is found in the fact that it is just as marked in the fetal intestine, which has not yet functioned, as in the adult animal. The permanence of the gradient in the gut is to be expected from studies on the lower forms of life. The mouth of a frog is lined by epithelium covered with little cilia which wave in one direction. If a piece of this epithelium is cut out, turned through an angle of 180°, and grafted back again, the cilia continue to beat in their original direction, now contrary to that in the rest of the mouth. In some of the worms the so-called "polarization" is so perfect that if the animal is cut into a half-dozen pieces they will all crawl in the same direction towards the point where the head used to be.

Before leaving the topic of rhythmic contraction I must make it clear that in the intestine the rhythm of one segment rarely influences the rhythm of the adjacent ones. As you know, in the heart the region with the fastest rate sets the pace for all others, and the wave of excitation travels so rapidly that to the naked eye the mammalian heart appears to contract simultaneously all over. In the stomach, the area with the most rapid rate at the cardia sets the pace, and we can see the waves traveling slowly to the pylorus. In the small intestine each segment contracts at its own rate and only occasionally do we see what are called peristaltic rushes running any distance down the bowel. Although the duodenal muscle has the fastest rate it does not set the pace for the rest of the gut. I must emphasize this point because some writers in overlooking it, have theorized unwarrantably on the basis of some purely anatomical observations reported by Keith.

UNDERLYING BASIS OF THE RHYTHMIC GRADIENT

The next question is: how are these differences in rate brought about? What are they due to? What are the structural differences behind them? As we have seen that these rhythmic movements are myogenic in origin, it is plain that we must look for the differences in the muscle. Now, it has been shown that the rate of rhythmic contraction is probably dependent upon the rate at which the chemical processes go on. Some substance is built up to a certain point and then exploded to produce the contraction. If the metabolism is slow it should take longer to complete the cycle. We know also that warming hastens chemical processes, and Taylor and I have shown that warming the intestine hastens the rate of rhythmic contraction. As we can take a piece of ileum beating 10 times a minute, and by warming it speed up its metabolism so that it will beat 17 times per minute, it seems to me that the duodenum which normally beats 17 times per minute must have a faster metabolic rate than the ileum. Such differences in metabolic rate have been demonstrated in other organs of the body, where they appear to have great significance.[12] During the last few years Miss Starkweather and I have brought forward a good deal of evidence to show that there is a gradient of oxidation from the cardia to the pylorus, from the duodenum to the ileum and from the ileocecal sphincter to the anus.[13] For the unit of weight and time the muscle from the duodenum gives off more CO_2 than does the muscle from the ileum. This is true not only for the active but also for the resting muscle. Graded differences have been found, moreover, in the catalase content of equal weights of minced muscle. This catalase, which liberates oxygen from hydrogen peroxid, is supposed by some to be an index of the rate of metabolism; and certainly in the bowel the gradients of CO_2 production and catalase activity run very close together. Be that as it may, the interesting thing to me is that we can show a definite chemical difference between the muscle in the duodenum and that in the ileum.[1]

We know that there is some gradation also in the irritability of the intestine as regards distension. The duodenum and jejunum are very responsive to the presence of food or balloons, while the lower ileum and colon are quite tolerant of them. You all know that the barium meal in the jejunum appears in small flecks as if it had been sprayed over the folds of the mucous membrane. In the ileum it forms dense sausage-shaped masses. That is due simply to the greater irritability and activity of the upper bowel.

Hess showed years ago that the pull exerted by the jejunum of a dog on a small balloon is 228 gm., while in the ileum it is 75 gm. Naturally, the food is going to move from the active and irritable regions to the more sluggish and insensitive ones. I cannot conceive of a simpler theory of peristalsis, or one more in harmony with the laws of physics and mechanics.

DIFFERENCES IN PERISTALSIS IN DIFFERENT PARTS OF THE GUT

Let us follow for a few moments the progress of a barium meal through the digestive tract, noting how the peristaltic movements are influenced by local differences in structure and musculature. The mouthful shoots through the first part of the esophagus because the muscle is quick-acting and striated. In the lower third the muscle is largely of the smooth variety and progress slows up. In the stomach the waves begin probably in the pacemaking region near the cardia and travel as shallow ripples until either proper pressure conditions or the presence of the peculiar antral muscle causes them to break into deep waves. If the tone of the stomach is too high the waves may be very shallow or hard to see; if the tone is poor, we may see the best waves at the beginning of the examination when there is only a little food present and the muscle fibers are not too badly stretched.

The waves do not cross the pylorus, probably because of the connective tissue barrier there, the peculiar arrangement of the muscle fibers, and the sudden transition to a different type of muscle. The control of the pylorus is partly chemical and partly mechanical. Cannon has shown that the presence of acid above tends to relax the sphincter and acid below tends to close it. We know however that this mechanism will not explain the vagaries of pyloric action in achylia gastrica, in duodenal ulcer, carcinoma, etc. We know also that the mere presence of food or of a distending balloon in the duodenum and jejunum will tend to stop the progress of food through the pylorus.[14] It is this mechanism that keeps the stomach from emptying too rapidly after pylorectomies, Mayo-Polya operations and gastro-enterostomies. This holding back above an active or distended part of the gut is easily explained on the basis of changes in the gradient. The digesting, stretched bowel has a faster metabolic rate and a more rapid rate of rhythmic contraction; hence the gradient is likely to be uphill towards it. This will tend to slow the progress of more food coming down from above.[15]

The duodenal cap remains filled and shows almost no peristalsis, probably because the muscle removed from that region shows very little rhythmicity. There is some evidence that the muscle fibers are arranged in festoons and not circularly and longitudinally, as they are elsewhere. This might also tend to modify the contractions. I think the peculiarities of the upper duodenal regions, together with a number of its pathological tendencies, can be traced back to the great specialization and complexity of this part of the bowel in some of the fishes.

As I have already stated, the jejunum is jejune or empty because of its great irritability and rapid peristaltic rate. The food slows up in the terminal ileum because the muscle is more sluggish and because the gradient is uphill for a short distance to the ileocecal sphincter.

In the first third of the colon the gradient is poor, so that the waves can go in either direction. In the rat they tend to be antiperistaltic when the feces are liquid and peristaltic when the feces become drier. The colonic contents move slowly because the muscle is more sluggish and perhaps because the gradient is poor. The tendency for the feces to stay out of the rectum can be explained if I am right in thinking that the gradient is uphill in that region. There is some evidence from animal experimentation in favor of that assumption.[16]

PRACTICAL VALUE OF THE GRADIENT THEORY

This idea of a gradient underlying peristalsis gives us facile explanations for many of the phenomena observed in disease. The gradient of forces can be steepened, flattened or reversed. A duodenal ulcer which raises the irritability and tone of the upper end of the tract often hurries the progress of food through the small intestine; a lesion in the appendix or cecum which raises the irritability of the lower end of the bowel slows the current and produces ileal stasis. A fissure in the rectum may cause back pressure into the cecum with constipation. A patch of enteritis in the jejunum can reverse the current above, in the duodenum and stomach—with vomiting; and can hurry it below—with diarrhoea. A stimulus reaching the jejunum from the brain by way of the vagi—as in sea-sickness—may also empty the tract both ways. The distension of any part of the tract by food raises the tone and irritability of that region and tends to hold back the material coming down from above.

Theoretically, the gradient can be reversed not only by raising the lower end, but by depressing the upper end. My observations on excised segments of intestine from distempered dogs and snuffling cats agree closely with those of Child on the lower forms of life in showing that the most sensitive regions, which have the fastest metabolic rates and the greatest need for oxygen, are injured most by asphyxia, by many drugs and by disease toxins. I have shown repeatedly that a poison can be administered in such dosage as to have no effect on the colon and ileum, while it paralyzes the duodenum and weakens the jejunum. Furthermore, exised segments of duodenum from a sickly rabbit may not contract at all in Locke's solution when similar segments from the ileum of that animal show no sign of toxic influence. A reversal or a flattening of the chemical gradients can also be shown in many of the sick animals. We have here, then, an easy and very simple explanation for the digestive upsets with intestinal stasis which we see in fevers and asthenic states.

As I have discussed the practical aspects of my theory in previous papers [17] I will not go into the subject any further at this time. Remember, when confronted by an abnormality in intestinal peristalsis, that if the lesion is sufficiently irritating it will raise the local tone; this will reverse the gradient leading to the lesion on the orad side and it will steepen the gradient on the caudad side. Hence it is that it may slow, stop or reverse the progress of material coming toward it from above, and may hasten the progress of material that has passed it. Exceptions to this rule will be found around the stomach where there are many complicating factors not sufficiently understood at present.

In closing, I must admit that with all the work that has been done we still know too little about the origin of many of the disturbances of function which we see in our patients—the hypo- and hypermotilities, the sphincter spasms and the peculiarities in gastric peristalsis. We must keep in mind that we are using a physiologic method, borrowed from Cannon; we must think more in terms of deranged physiology, and must not rest too satisfied with the demonstration of beautiful morphologic defects—ulcer craters, carcinomas, adhesions and displacements. A number of you—practising radiographers—have made excellent physiologic studies on man; studies that are as much pure physiology as those of Cannon and Carlson. Much remains to be done; and if my little message from the biologists to-day should contribute anything to your zeal and skill in attacking these problems I shall be happy.

SUMMARY

We often fail in trying to solve our practical problems because we do not begin near enough to the beginning. We must know more about general principles and the workings of small parts of the digestive tube.

We must venture out more into "pure" science. We can get much help and inspiration from the experimental zoologists and the physiologists.

The digestive tract is largely autonomous, and the forces underlying peristalsis must be looked for mainly within the gut itself.

The tendency of writers to explain everything on the basis of nervous reflexes and ganglionic control is unfortunate, and paralyzing to further progress.

Recent biologic work has shown in numberless ways that the main function of the nervous system is conduction.

It has been shown conclusively that the rhythmical contractions of the intestinal muscle are myogenic in origin.

Auerbach's plexus serves to conduct stimuli and to coordinate the activities of different parts of the tract. It contains no reflex arcs.

Current theories about the antagonism between the vagus and sympathetic are shown to be pseudoscientific, and out of harmony with the facts as determined by the leading anatomists and physiologists of the world to-day. The sympathetic is an integral part of the central nervous system and not an outlying antagonistic "brain."

Many of the conclusions based upon the supposed actions of drugs on nerve-endings and "intermediate substances" must be revised in the light of recent work.

The extrinsic nerves of the intestine serve to communicate between the body and the bowel, and between the bowel and the brain.

The digestive tract can work quite normally after section of all the extrinsic nerves.

The key to an understanding of peristalsis is to be found in a study of the smooth muscle in the wall of the bowel.

There are different types of muscle suited to different functions in the different parts of the body and in different parts of the digestive tract.

The properties of smooth muscle are discussed: its reactions to stimulation, to distension, inflammation, etc.

A peristaltic wave spreads both ways from a stimulated spot like ripples from a stone thrown into a pond. Most of the muscular tubes are so constructed that the ripples can travel in one direction better than another.

It may perhaps be a law for all tubular organs that the transport of material in one direction depends upon gradients of rhythmicity, irritability, tone and metabolism.

Such gradients can easily be demonstrated in the walls of the stomach and intestine.

A bolus moves aborally in the digestive tract because the pressure is greater on the upper than on the lower side. This difference in pressure is due probably to the graded characteristics of the muscle.

The peculiarities of peristalsis in different parts of the tract are explainable on the basis of local differences in the structure of the neuromuscular mechanism.

It is shown how the gradients may be upset in disease, and how these theories can explain pathologic phenomena.

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THE USE OF CO_2 IN PNEUMOPERITONEUM

IX

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THE USE OF CO_2 IN PNEUMOPERITONEUM*

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A YEAR ago, when I began experimenting with pneumoperitoneum, it seemed to me that it would never come into general use unless some way were found of relieving the patient's discomfort immediately after the exposures were made. I felt that the technique would have to be modified in some way so that we could do the work in the office and not in the hospital. Some studies on the absorption of gas from the intestinal cavity had made me acquainted with the fact that CO_2 is absorbed much more rapidly than oxygen. In July, 1919, Dr. Taylor and I injected the preitoneal cavities of a number of rabbits with CO_2 and found that it was absorbed in a few minutes. After convincing ourselves that this gas was as harmless as oxygen we started using it on patients in the office. We found that whereas oxygen often remains in the abdomen in sufficient amounts to cause distress for four days after injection, the CO_2 in no instance remained over half an hour. Ordinarily the patient was relieved of tension in twenty-five minutes.

Dr. Stewart has stated in a recent article that he has tried to make the procedure suitable for office practice by withdrawing the gas through a trocar after the plates are taken. My experience with these patients makes me very doubtful whether many of them would be willing to submit to a second puncture while they are in pain. Moreover, I would rather not make another wound, although my experience with patients who have been operated upon after inflation is in accordance with that of others who have found no sign of damage. In only one case did I see a light strand of omentum adhering to the scar. Another objection to deflation is that it is hard to get all the gas out, and unless this is done completely the patient is going to remain uneasy.

TECHNIQUE

The introduction of the gas is simplicity itself. I use as a measuring bag the rubber cuff that comes with a sphygmomanometer. This holds about a liter. It is connected with the CO_2 tank and with the needle. The air in the tubing should be washed out with CO_2 because the nitrogen in it will remain in the abdomen for 10 days or more. No attempt need be made to sterilize the gas. The needle is sterilized and then thrust through a small spot which has been painted with iodine. I think there is no need for using novocain unless a very large trocar is employed. I use a needle with a caliber slightly larger than that of a lumbar puncture needle. I always clear it first by injecting a few drops of sterile salt solution. The injection is made generally at a point in the middle of the left rectus near the navel. I find it hard to get the patients to take enough of the gas. It is easy with old women who have lost weight and who have flaccid abdominal walls, but it is very hard with muscular men. I make it a routine to give a quarter of a grain of morphin a half hour before the injection. This unfortunately often produces nausea and vomiting later, but it seems the lesser of two evils because one never can tell which patients are going to complain bitterly. Even with the morphin, some do complain of pain, particularly in the right shoulder. Others do not complain of much pain but break out into a cold sweat and seem to be greatly distressed mentally. Others again have paid very little attention to the procedure, and one man got off the table and went out to lunch with me.

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I think the best evidence that the operation is not a terrible one is the fact that two of my patients, who did not take quite enough gas the first time, volunteered to have the work repeated.

The only disadvantage that I can see to the CO_2 technique is that one must work rapidly if a number of plates are to be secured. We almost always take first a 14 x 17 plate with the patient lying prone. This is developed immediately so that any special points of interest can be focused on in other plates. It is very helpful when the patient has not much gas in his abdomen to rotate him slightly so that the right side is a little higher for the gall-bladder plates and the left side a little higher for the spleen plates. In this way one makes the most of the gas that is there.

I made a few attempts to use mixtures of oxygen and CO_2, hoping that I might slow down the deflation somewhat, but I promptly gave this up because all the disadvantages of oxygen were retained without sufficient compensatory advantages from the CO_2.

Just a word as to the usefulness of the method. If it were not for the distress to the patient one would like to have a set of these beautiful and instructive plates on every individual who comes with intra-abdominal troubles. Unfortunately, however, the procedure is so alarming and distressing to many patients that I do not believe it can ever be used as a routine. I have found it of most help in the diagnosis of gall-bladder disease, especially in the early stages. I believe that daily we are slipping up on the diagnosis of early cholecystitis. We should be helping these people before they develop more definite symptoms, before they get stones and before they suffer irreparable damage to the liver and pancreas. One of the remarkable things about this technique is that it opens up a clear space between the liver and the kidney. Normally, the bowel should drop down out of this space; and in a number of cases in which it did not do that, operation showed the expected adhesions and the expected gall-bladder disease.

In this way I have been able to help a number of women who otherwise would have gone on suffering, because I would not have had the courage to advise operation in the absence of most of the classical signs of cholecystitis. Although some of the thickened gall-bladders without stones show up very clearly on these plates, it is most disappointing that others do not. I have here to-day a plate which fails to show any gall-bladder shadow although it does show a stone which gave symptoms for twenty-five years. To be sure, operation showed that the wall of the gall-bladder in this case was still thin. Moreover, in spite of repeated attacks of inflammation there were no adhesions to the colon. In other cases it was hard to be sure of the disease in the gall-bladder, even at operation. The wall was not definitely thickened, the bile was sterile, there were only a few adhesions to the duodenum and a large gland on the cystic duct. Nevertheless the pathologist reported small abscesses, full of bacteria, and the patient got well after the cholecystectomy. I think these observations must keep us humble, and must make us admit that the roentgen ray diagnosis of early cholecystitis will often be impossible and always difficult and uncertain.

In spite of these disappointments I still feel that the gas technique is going to be very useful. When the history and other findings point strongly to cholecystitis, and when a series of plates, taken in the usual way, leave the question undecided, I practically always resort now to this technique and often get a definite answer. For a while, after discovering the value of CO_2, I kept using O_2 for special cases; but the differences in the distress of the two groups of patients was so pronounced that I finally gave up the use of oxygen entirely. I think that others who compare the after effects of the two gases will come to the same conclusion, and that the use of CO_2 will win out, unless some one can find another gas which is absorbed in perhaps forty minutes. Even then, CO_2 might remain the gas of choice because it is cheap and a common article of commerce.

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X.

PROGRESS REPORT ON THE INVESTIGATION OF THE CHEMOTHERAPEUTICS OF CHAULMOOGRIC ACIDS IN TUBERCULOSIS*

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San Francisco

I. BASIS OF THE INVESTIGATION

Chaulmoogra oil has had an empiric reputation in the Orient for the treatment of leprosy since ancient times. In order to determine whether or not there existed any scientific grounds for this reputation, an investigation of the therapeutic properties of this oil was undertaken in my laboratory in 1918. This was first directed to a study of the method of action, active principle and specificity of chaulmoogra oil. The more important conclusions from this part of the investigation, which was published in the *Journal of Infectious Diseases,* March, 1920, are as follows:

1. Chaulmoogra oil contains substances having a high bactercidal activity.

2. This bactericidal actvity is a property of a series of fatty acids, having a carbon ring structure and peculiar to chaulmoogra oil and oils from certain plants closely related to Hydnocarpus (Taraktogenos) kurzii.

3. The bactericidal activity of the chaulmoogric acids is specific for the acid-fast groups of bacteria, and inactive against all other bacteria.

Meanwhile clinicians, using the salts and esters of chaulmoogric acids, have apparently demonstrated conclusively the curative action of these cyclic fatty acids in leprosy. These therapeutic results have been obtained especially by Sir Leonard Rogers and his colleagues in India with the sodium salts administered intravenously, and by MacDonald, Dean and Hollmann, in Hawaii, with the ethyl esters administered intramuscularly. These latter authors report the parole of 142 patients apparently cured of leprosy during the past two and one-half years.

These two facts, the specific bactericidal action of the chaulmoogric acids on acid-fast bacilli and the therapeutic results obtained by their use in one of the diseases caused by acid-fast bacilli, are the basis of this investigation of the chemotherapeutics of the chaulmoogric acids in tuberculosis.

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* From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco.

Work done under grant of the Committee on Medical Research of the National Tuberculosis Association.

II. SOME PRELIMINARY EXPERIMENTS ON THE TREATMENT OF TUBERCULOSIS IN LABORATORY ANIMALS WITH CHAULMOOGRA OIL AND ITS DERIVATIVES

The use of laboratory animals, such as guinea pigs and rabbits, for the experimental therapy of tuberculosis has its advantages and its disadvantages. Tuberculosis in man is not necessarily a fatal disease and spontaneous arrest and cure of the disease is not uncommon (and is perhaps the rule, judging from our knowledge of childhood infections and of autopsy findings) ; but in guinea pigs and rabbits, infected with a strain of known virulence, the disease runs an invariably fatal course. Consequently, cure or even consistent prolongation of the average life of the treated beyond that of the control animals in therapeutic experiments can be ascribed with reasonable certainty to the treatment. This is a distinct advantage that laboratory animals have over human patients for such experiments. On the other hand, there is this definite disadvantage that tuberculosis is a more acute disease in laboratory animals than in man and there is consequently less time for treatment and, other things being equal, treatment would have to be more efficient to obtain cure of these animals. This is particularly disadvantageous in the case of a slow-acting therapeutic agent like chaulmoogric acids; for our experience in the treatment of leprosy has shown that the chaulmoogric acids act very slowly, and that from one to two years' treatment is necessary to effect a cure. The life of a tuberculous guinea pig or rabbit is much less than this time, and is usually measured by one to a few months. Possibly the most that we can hope to accomplish under these conditions is to modify to some degree the course of the disease and obtain a consistent prolongation of life of the treated animals.

In these preliminary experiments, many factors have contributed to impair the results and preclude drawing definite conclusions. Too few animals were used in most of these experiments, and the number was still further reduced in many cases by deaths from intercurrent infections and by premature sacrifice of animals. Rabbits especially, when their resistance is lowered by tubercular infection and by inoculation treatment, are very susceptible to infection with *bacillus cuniculisepticus,* giving rise to pneumonia, empyema, etc., and this infection spreads from rabbit to rabbit if they are not kept in individual pens. Treated animals, that did not succumb to intercurrent infections and outlived the controls, were unfortunately sacrificed in many cases, either to determine the course of the disease or because it was believed that they had failed to become infected. In the earlier experiments, too large doses of tubercle bacilli were inoculated, which gave overwhelming infections that ran a too acute course for successful treatment. And, in the light of our knowledge of the slow therapeutic action of the chaulmoogrates in leprosy, treatment of the animals in these experiments was not sufficiently intensive and prolonged to obtain maximum results.

The general method of conducting these experiments has been to infect a certain number of animals with a uniform quantity of a virulent strain of human tubercle bacilli, to treat half of the animals and reserve the other half as controls. Treatment was begun usually on the day of infection, because the life of a tuberculous guinea pig or rabbit is relatively so short and the possible therapeutic action of the chaulmoogrates probably so slow that otherwise no results could be expected, and because it was the purpose of these preliminary experiments to determine whether or not chaulmoogra oil or its derivatives were capable, under the most favorable conditions, of modifying to any appreciable degree the course of tuberculosis in the experimental animals. In a few experiments, however, one to four weeks intervened between the infection and treatment of the animals. The effect of treatment was estimated, after excluding animals that died from intercurrent infections or other accidents, by the average life of the treated as compared with that of the control animals and by the findings at necropsy. In the following table are given the essential data of these experiments.

From these preliminary experiments, owing to the small number of animals used and to various accidents that interrupted them, it would be unwise to draw any hard and fast conclusions; all that I am willing to say of them is that they are suggestive. They suggest, first, that chaulmoogra oil and its derivatives have some therapeutic action on experimental tuberculosis, which is partly masked by the slowness of this action and the relatively acute course of the disease in these animals. Although premature deaths from secondary infections and other causes reduced the number of the treated more than of the control animals, nevertheless the average length of life of the treated exceeded that of the control animals in ten out of the eleven experiments. Many of the treated animals which outlived the controls were killed in good condition and showed lesions that were apparently retrogressive or no lesions. These retrogressive lesions consisted of connective tissue containing little or no caseous material, and sometimes of empty connective tissue sacs. There is, of course, a temptation to regard the absence of tubercular lesions in some of these animals, in which treatment was begun immediately after infection, as due to a prophylactic action of the early treatment; but I doubt if such a conclusion would be warranted in so small a series of animals. It is noteworthy, however, that all of these animals without tubercular lesions fall in the treated groups. Secondly, these experiments indicate that, as in leprosy, the therapeutic action of chaulmoogra oil and such derivatives as have been used is slow in tuberculosis and that treatment must be continued a long time to obtain results. And, thirdly, it would appear, as is also observed in leprosy, that not all tubercular animals respond equally to the treatment. Some of the treated animals went steadily downward and died of tuberculosis at about the same time as the controls; others lived much longer than the controls, were killed in good condition, and showed apparently retrogressive lesions or no lesions. However, it is recognized

TABLE I

Treatment of Experimental Tuberculosis with Chaulmoogra Oil and its Derivatives.

EXPERIMENT NUMBER	ANIMALS	TREATMENT	FATE OF ANIMALS	NECROPSY FINDINGS	AVERAGE LENGTH OF LIFE IN DAYS
1	5 guinea pigs.	Heiser's mixture of chaulmoogra oil 1:75,000 to 1:10,000 of body weight, subcutaneous, at 3-5 day intervals.	Died.	T. B.	71
	5 guinea pigs.	Controls.	Died.	T. B.	58
2	5 guinea pigs.	Heiser's mixture of chaulmoogra oil, 1:20,000 to 1:5,000 of body weight, subcutaneous, at 5-7 day intervals.	Died.	T. B.	45
	5 guinea pigs.	Controls.	Died.	T. B.	36
3	3 guinea pigs.*	Heiser's mixture stained with Sudan III 1:25,000 to 1:15,000 of body weight, subcutaneous, at 4-7 day intervals.	2 died. 1 killed, 113th day.	T. B.	90+
	1 guinea pig.	Control.	Died.	T. B.	72
4	5 rabbits.	Sodium salts of chaulmoogric acids, 1:300,000 to 1:100,000 of body weight, intravenous, at 4-5 day intervals, 6 treatments only.	1 died of secondary infection, 49th day. 4 killed 134-145th day.	T. B. Empyema. T. B. 1 retrogressive? 3 progressive.	139+
	5 rabbits.	Controls.	2 died T. B. 3 killed 134-145th day.	T. B. All progessive.	99+
5	2 rabbits.	Sodium salts of chaulmoogric acids, 1:750,000 to 1:100,000 of body weight, intravenous, at 4-7 day intervals, 22 treatments.	Both killed 151st day.	Both negative	—
	1 rabbit.	Emulsified chaulmoogra oil, 1:750,000 to 1:100,000 of body weight, intravenous, at 4-7 day intervals, 22 treatments.	Killed 151st day.	T. B. A single focus (4-5 m.m.) in right lung, retrogressive?	—
	2 rabbits.	Controls.	Both killed 151st day.	T. B. progressive.	—

TABLE I — Continued

EXPERIMENT NUMBER	ANIMALS	TREATMENT	FATE OF ANIMALS	NECROPSY FINDINGS	AVERAGE LENGTH OF LIFE IN DAYS
6	3 rabbits.	Sodium salts of chaulmoogric acids, 1:500,000 to 1:100,000 of body weight at 3-7 day intervals, treatment started after one month.	1 died of secondary infection 4th day. 1 killed 140th day. 1 died 222nd day.	Empyema. T. B. progressive. T. B. retrogressive?	151+
	3 rabbits.	Controls.	Died.	T. B. progressive.	86
7	3 rabbits.	Sodium salts of chaulmoogric acids, 1:500,000 to 1:100,000 of body weight, intravenous, at 4-7 day intervals, 13-24 treatments.	1 died of secondary infection 13th day. 1 died of strangulation 71st day. 1 killed 168th day.	Empyema. T. B. retrogressive? T. B. retrogressive?	123+
	3 rabbits.	Controls.	1 died of secondary infection 5th day. 1 died T. B. 78th day. 1 killed 168th day.	Empyema. T. B. progressive. T. B. progressive.	119+
8	3 rabbits.	Sodium salts of chaulmoogric acids, 1:500,000 to 1:100,000 of body weight, treatment started after 7 days, at 7 day intervals 24-35 treatments.	1 became paralyzed and was killed 171st day. 2 died.	T. B. retrogressive? Both T. B. progressive.	231+
	3 rabbits.	Controls.	Died.	T. B. progressive.	256
9	3 rabbits.	Sodium salts of chaulmoogric acids, 1:500,000 to 1:100,000 of body weight, intravenous treatment started after 14 days, at 5-8 day intervals, 28-34 treatments.	1 died of secondary infection. 2 died of T. B.	Empyema and lung abscess. Both T. B. progressive.	234
	3 rabbits.	Controls.	1 died of secondary infection. 2 died of T. B.	Empyema and lung abscess. T. B. progressive.	148

TABLE I — Concluded

EXPERIMENT NUMBER	ANIMALS	TREATMENT	FATE OF ANIMALS	NECROPSY FINDINGS	AVERAGE LENGTH OF LIFE IN DAYS
10	3 rabbits.	Sodium salts of chaulmoogric acids, 1:500,000 to 1:100,000 of body weight, intravenous, at 2-7 day intervals, 22-32 treatments.	1 became paralyzed and was killed 95th day. 1 killed in good condition 369th day. 1 became paralyzed and was killed 446th day.	All T. B. retrogressive?	303+
	3 rabbits.	Controls.	1 died of secondary infection. 2 died of T. B.	T. B. progressive.	207
11	4 rabbits.	Ethyl chaulmoogrates intramuscular, 0.01 cc.-0.1 cc., at 3-7 day intervals, 18-28 treatments.	2 became paralyzed and were killed. 2 died, 1 with abscess of thigh.	T. B. progressive. negative. T. B. retrogressive? T. B. progressive.	165+
	4 rabbits.	Ethyl chaulmoogrates emulsion, intravenous, 1:500,000 to 1:100,000 of body weight, at 3-7 day intervals, 13-29 treatments.	1 died 15th day, cause unknown, 1 died suddenly after injection, 72d day. killed 203d day in good condition. 1 still alive on 424th day.	— T. B. progressive. T. B. retrogressive? — —	236+
	4 rabbits	Controls.	Died.	T. B. progressive.	95

*This experiment was to study absorption of Heiser's mixture, stained with Sudan III.

that a more extended series of experiments may completely overturn these tentative conclusions.

III. WORK IN PROGRESS

The work now being undertaken on this problem may be discussed conveniently under three heads: (1) the preparation of derivatives of chaulmoogric acids; (2) biochemical studies of the absorption, distribution and fate of chaulmoogra oil and its derivatives in the animal body, and (3) therapeutic tests of the chaulmoogric acids and their derivatives on animals experimentally infected with tuberculosis.

The purpose of the synthetic work on the chaulmoogric acids is to obtain, if possible, a compound which will be more rapidly absorbed, will not be decomposed and stored in the tissues as glycerol esters or fat, will penetrate the tubercular lesions, and will have a more rapid and intensive therapeutic action than those hitherto used in leprosy. The preparation of these derivatives is limited by the physical and chemical properties of these fatty acids, which render many of their compounds unsuitable for therapeutic use. Of the salts, only those of sodium and potassium are water soluble and suitable for intravenous injection. The esters of chaulmoogric acids, after vacuum distillation, are thin oily fluids suitable for intramuscular injection. These esters, containing alkyl radicles, are of considerable interest to us because of the superior therapeutic results obtained with one of them in leprosy, and on account of the observations of several investigators on the effect of alkyl radicles introduced into bactericidal compounds. It is well known that the introduction of an alkyl radicle into phenols, forming cresols, enhances the bactericidal properties of these compounds. Furthermore, Churchman (1913), Jacobs, Heidelberger and Bull (1916), Kleigler (1918), Crossley (1919) and Morgenroth and Kauffmann (1920) have shown that the bacteriostatic and bactericidal actions of other organic compounds are augmented by the introduction into them of alkyl radicles. Kleigler found that an increase in the number of alkyl radicles increased the bacteriostatic action and that the bacteriostatic action is enhanced to a greater degree by the ethyl than by the methyl radicle. Jacobs, Heidelberg and Bull concluded that in the hexamethylenetetramin compounds the bactericidal activity increased from the methyl to the ethyl to the propyl compound, that is, was proportional to the molecular weight of the introduced alkyl radicle. Morgenroth and Kauffmann, however, found in the hydrocupreine compounds that, while alkyl radicles caused an increased bactericidal action against pneumococci and that this increase varied with the different alkyl radicles, the variation did not strictly correspond to their molecular weights. Thus propylhydrocupreine was less active than ethylhydrocupreine, but isoamylhydrocupreine had a markedly increased bactericidal action. Because of the superior therapeutic results obtained in leprosy with the ethyl esters of chaulmoogric acids and the possible enhancing effect of certain higher alkyl radicles on bactericidal action, it has seemed especially desirable to prepare for therapeutic test the whole series of alaphatic esters and all of their isomers of chaulmoogric acids. Concerning the possibilities of the aromatic esters of chaulmoogric acids, nothing is known. It is of interest, however, that Macht, Satani and Swartz (1920) have shown that benzyl alcohol, even in considerable dilutions, has pronounced bactericidal properties; that Jacobson (1919) has found that benzyl alcohol has a high capacity to dissolve tubercle bacilli *in vitro;* that Jacobs, Heidelberg and Bull (1916) found the benzylethyl derivative of hexamethylenetetramin eminently bactericidal; and that many of the benzine derivatives possess germicidal properties or are capable of enhancing the germicidal properties of other substances with which they are combined. All of the above are examples of compounds, which we are preparing, having the introduced element or radicle attached to the side chain of the chaulmoogric ring. The introduction of elements or radicles into the ring of chaulmoogric acid offers almost unlimited possibilities of modfying the structure and possibly the therapeutic action of this acid.

The more important biochemical problems presented by this investigation are: (1) the absorption of chaulmoogra oil and its derivatives from the alimentary tract, the tissues and the blood; (2) do any of the derivatives of chaulmoogric acids, when inoculated into the blood or tissues, circulate in the blood unchanged? or (3) are the chaulmoogric acids, in whatever form and way they may be introduced into the animal body, combined with glycerol and stored in the tissues as fat? (4) are any of the derivatives of chaulmoogric acids able to penetrate the tubercular lesions and reach the tubercle bacilli? and (5) what is the ultimate fate of chaulmoogric acids in the animal body?

If chaulmoogra oil or its fatty acids are absorbed readily from the alimentary tract, it would seem that ingestion of them would be a convenient method of at least supplementing the injections to secure saturation of the body with the therapeutic agent. However, McDonald and Dean have decided that no better or more rapid therapeutic results are obtained in leprosy by supplementing the injections of ethyl esters by ingestion of the fatty acids, and the old method of administering the whole oil by mouth apparently gave therapeutic results in leprosy much inferior to the present method of intravenous or intramuscular injections of the salts or esters. Absorbed from the alimentary tract chaulmoogra oil, or its fatty acids, undoubtedly is stored in the tissues as glycerol esters, like other fats, and probably can circulate in the blood and reach the tubercular lesions, if at all, only when metabolized into phospholipoids such as lecethin. This would perhaps explain the slow action of chaulmoogra oil administered by mouth in leprosy, as it would probably be necessary to replace to a certain extent the normal fats of the body with glycerol esters of chaulmoogric acids before a sufficient amount of phospholipoids containing these fatty acids can be turned into the circulation to exercise a therapeutic action. If chaulmoogric acid compounds injected into the blood or tissues are invariably decomposed and stored as fat, then these derivatives and their method of administration would appear to have no advantage over the ingestion of the whole oil; but, if some of them circulate in the blood intact, the hope of discovering some derivative having a more rapid and intense therapeutic action would be well grounded. The fact that the sodium salts and ethyl esters intravenously or intramuscularly have given therapeutic results in leprosy much superior to the ingestion of the whole oil or its fatty acids lends support to this hope. Finally, the therapeutic agent must not only circulate in the blood, but it must be able to penetrate the tubercular lesions in order to exercise its bactericidal action. The penetration of tubercular lesions with their more or less extensive areas of necrotic tissue deprived of blood supply is probably quite a different matter from the penetration of leprous lesions, and may mean all the difference between success in the therapy of leprosy and failure in tuberculosis.

The animal experiments are being undertaken on a large scale; and every effort is made to correct the shortcomings of our preliminary experiments and to minimize the handicap of slow therapeutic action of the chaulmoogrates and short life of the experimental animals. These experiments are directed, first, to determine definitely whether or not chaulmoogric acids are capable of inhibiting or modifying the course of experimental tuberculosis. Our preliminary experiments, it is true, suggest that the chaulmoogrates do have some therapeutic action, which is in part masked by the slowness of this action and the acute course of the disease in the experimental animal; but a larger series of carefully controlled experiments are necessary to establish the fact or fallacy of this impression. Secondly, these experiments are intended to test the relative therapeutic activity of the large series of derivaties of chaulmoogric acids, which we are preparing, with the hope of discovering one having more rapid and intensive action. Such comparative tests, even if they fail to demonstrate among our derivatives one of greatly superior therapeutic properties, may at least indicate the direction which further synthetic work should proceed to attain this end. Other important points to be determined by these animal experiments are the relative efficacy of administration of chaulmoogra derivatives by mouth, by intramuscular and by intravenous injections, dosage, toxicity, and prophylatic action.

It is hoped that the work now under way will, when completed, give us sufficient data from which to draw definite conclusions as to the therapeutic possibilities of chaulmoogra oil derivatives in tuberculosis; conclusions that will either preclude the necessity of more work on the problem, or will stimulate further investigation. Should we fail to attain the full measure of success hoped for in the therapy of tuberculosis, it will be a source of some satisfaction to all of us, I believe, to know that the information obtained in this investigation will be to a large extent applicable to leprosy and should contribute materially to the successful treatment of this disease.

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XI

Further Observations on the Clinical Value of Certain Bacteriostatic Triphenylmethane Derivatives

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FURTHER OBSERVATIONS ON THE CLINICAL VALUE OF CERTAIN BACTERIOSTATIC TRIPHENYLMETHANE DERIVATIVES

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The specificity of the action of certain drugs against particular infections has been recognized for a great many years. Perhaps one of the first drugs to be so used was quinin, which was employed in its original state in the treatment of malaria, between the years 1630 and 1640. In 1638, it is said to have proved efficacious in the case of the Countess of Chinchon, whose name was later applied to the bark which the Spanish brought from South America.

After an exhaustive investigation of certain arsenic compounds, Ehrlich[1] arrived at the formula of arsphenamin in the treatment of syphilis. In this case, the toxophore radical of arsphenamin has a greater affinity for the parasite than it has for the body cells of the animal host.

These two classical cases may be cited as opening the way to further knowledge of specific chemotherapy.

CHEMISTRY OF DYESTUFFS

I shall consider first the chemistry of certain dyestuffs, whose action will be discussed in more detail later.

Triphenylmethane, $CH(C_6H_5)_3$, is a crystalline substance and serves as the base for many of the dyes. There are three stages in the formation of the dye: (1) the formation of a (colorless) leukobase; (2) the formation of a (colorless) color base, and (3) the formation of a dye.

Many dyes exist as salts, but on account of their basic reaction they readily dissolve in acid solutions, losing their characteristic color which reappears when an excess of alkali is added to neutralize the acid.

Pararosanilin and rosanilin may have several of the hydrogen atoms of the amino groups replaced by alkyl radicals. The simplest of these dyes are yellow in color, and with the increase in molecular weight, or, in other words, with the increase in the number of alkyl or phenyl groups, the color is changed through orange and red to violet and blue. These substances are crystalline in form, and a great many of them are insoluble in water but highly soluble in alcohol.

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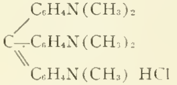
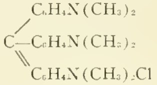
[1] Ehrlich: Abhandlungen über Salvarsan **1-3**.

It is possible to create certain intensities in color by the addition of certain radicals to the general formula. The addition of methyl groups increases the violet color in direct proportion to the number of methyl groups added. Thus the double salt, pentamethylpararosanilin and hexamethylpararosanilin or "methyl violet" is formed, whose structural formula is a mixture of the following:



$$C\begin{cases} C_6H_4N(CH_3)_2 \\ C_6H_4N(CH_3)_2 \\ C_6H_4N(CH_3)\ HCl \end{cases}$$

and



$$C\begin{cases} C_6H_4N(CH_3)_2 \\ C_6H_4N(CH_3)_2 \\ C_6H_4N(CH_3)_2Cl \end{cases}$$

By the replacement of one hydrogen atom of each amino group of rosanilin by a phenyl radical, a blue dye may be formed; thus we may make "anilin blue," triphenylrosalin-hydrochlorid. The intensity of the blue color depends here also upon the number of phenyl groups added to the structural formula.

Crystal violet (hexamethyltriaminotriphenylmethane) serves as an excellent example of a phenomenon exhibited by an analogous group of basic substances. When an equivalent amount of alkali is added to any salt of crystal violet, there is no immediate change in the color of the compound. But with the addition of a similar amount of acid, the color base dissolves to form a compound of a different color and not highly water soluble. This color base can, however, be reprecipitated by the addition of an equivalent amount of alkali to the solution, thus bringing back the violet color.

The foregoing will give the reader some idea of the relationship of these compounds to one another and show how the triphenylmethane radical is the fundamental basis of this entire group of compounds.

INHIBITORY PROPERTIES OF DYESTUFFS

Many of these substances have long been known to exhibit inhibitory properties upon the growth of certain organisms in culture mediums. Peabody and Pratt[2] used malachite green solution as an enriching agent in the isolation of *B. typhosus*.

Churchman,[3] found that gentian violet in dilutions of 1:100,000 inhibited the growth of certain types of bacteria while other types were not affected by its presence. Those organisms which grew in the presence of gentian violet solution were of the gram-negative type, whereas those which were inhibited belonged to the gram-positive group. Churchman [4] used a diagram to illustrate the effect of gentian violet upon gram-negative and gram-positive organisms. He found that out of 182 strains of gram-positive organisms, only seventeen strains were unaffected, and out of 136 strains of gram-negative organisms, only fifteen were unaffected by the dye.

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[2] Peabody, F. W., and Pratt, J. H.: On the Value of Malachite Green Media in Differentiating Typhoid and Colon Bacilli with the Description of a New Method of Isolating Typhoid Bacilli from Feces, Boston M. & S. J. **158:** 213, 1908.

[3] Churchman, J. W.: The Relative Bactericidal Action of Gentian Violet, J. Exper. M. **16**:221, 1912. Churchman, J. W., and Michael, W. H.: The Selective Action of Gentian Violet and Closely Related Bacterial Stains. J. Exper. M. **16**:822, 1912.

In an earlier publication, he used the terms "violet-positive" and "violet-negative" for the action of the dye in its inhibition as associated with gram-positive and gram-negative organisms. This nomenclature was objected to on the ground that the selective action of the dye is not at all dependent upon the color of the dye but rather upon some other factor. Consequently, he modified the terms in regard to the use of gentian violet to "gentian-violet-positive" and "gentian-violet-negative" in regard to its selective action upon the growth of bacteria.

Since, however, these terms are only adaptable to the one dye mentioned above, they could hardly be applied to other dyes of the same type. Better terms would be perhaps "triphenylmethane-positive" and "triphenylmethane-negative" in regard to the action of this whole group of chemicals in their selectivity upon the growth of gram-positive and gram-negative organisms.

Crossley [5] also noted that the inhibition of gram-positive organisms by crystal violet took place when other members of the triphenylmethane group were used.

He assumed that there are two factors at work which control the action of the dye: (1) the composition of the dye, and (2) the nature of the organism. He further suggested that there must be certain groupings inherent in the dye which combine with certain "reactive groups" in the structure of the organism, and thus produce bacteriostasis.

This selective action appears to be related in some way to the basic nitrogen, and substitution of methyl grouping for the aminohydrogen groupings causes an increase in bacteriostasis on the part of the compound. He called attention to the value of this feature in the treatment of infections, and suggested that further work be done on allied compounds.

It was Churchman [6] who called attention to the fact that dahlia, parafuchsin, magenta, pararosanilin, rosanilin, crystal violet, methyl violet, all have one thing in common, that is, their selective action upon bacteria, and he assumes that since each compound is a derivative of triphenylmethane, the power of bacteriostasis is inherent in this triphenylmethane radical.

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[4] Churchman, J. W.: Gentian Violet in the Treatment of Purulent Arthritis, J. A. M. A. **75**:583 (Aug. 28) 1920.

[5] Crossley, M. L.: Gentian Violet, Its Selective Bactericidal Action, J. Am. Chem. Soc. **41**:2, 2083, 1919.

[6] Churchman, J. W.: The Selective Bactericidal Action of Stains Closely Allied to Gentian Violet, J. Exper. M. **17**:373 (April) 1913.

Churchman,[7] in explaining the mechanism of this parallelism, states that "the factor which determines the relation of an organism to the gram process of staining is not the same as the factor which determines its relation upon divided gentian violet plates or after staining with the dye."

Nevertheless, combined with the gram stain, a "gentian" positive or "triphenylmethane" positive dye contains certain groups possibly related, as Crossley states, to the substituted alkyl radicals for the aminohydrogen of the basic nitrogen. These combinations when added to the "reactive groups" of the structure of the organism combine with them and later are fixed by the iodin solution which acts as a mordant and prevents the redissolution of the dye. This process definitely takes place in fixed or dead organisms. The same reaction may occur in the instance of living organisms, save that here the combination of dye with the "reactive groups" of the organism results not in mere staining of the parasite, but prevents further metabolism and division on the part of the bacterium by effecting some change in the tissue structure of the organism. At any rate, this idea must be kept in mind when considering the action of this group of substances.

Krumwiede and Pratt [8] not only verify the statements of Churchman, but state that occasionally there is found a particular strain of a gram-negative organism, which will not grow upon gentian violet mediums. This strain of organism was found to differ from other strains of the same organism by agglutination tests, which may account for the noninhibition in the same dilution of dye.

Krumwiede [9] recently made a brilliant green agar for the isolation of typhoid bacilli. Brilliant green, a diaminotri-phenylmethane derivative, they found not only inhibited gram-negative organisms but also many types of gram-positive bacteria as well. It exerted a specific differential action upon the colon-typhoid group. *B. lactis aerogenes* and the paratyphoid organisms are not affected, and *B. typhosus* is inhibited only in lower dilutions, whereas dysentery and the other colon groups are inhibited.

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[7] Churchman, J. W.: The Cause of the Parallelism Between the Gram Reaction and the Gentian Violet Reaction, Proc. Soc. Exper. Biol. & M. **18**: 17, 1920.

[8] Krumwiede, Charles, and Pratt, J. H.: Observations on the Growth of Bacteria on Media Containing Various Aniline Dyes, Proc. New York Path. Soc. **13**:43, 1913; Dahlia Agar als Unterscheidungs Mittel Zwischen Cholera und Anderen Vibrionen, Centralbl. f. Bakteriol. **58**:585, 1913.

[9] Krumwiede, Charles: Footnote 8. Hiss, P. H., and Zinsser, H.: Textbook of Bacteriology, New York, D. Appleton & Co., 1919.

APPLICATION OF SPECIFIC CHEMOTHERAPY TO INFECTED WOUNDS

Such information as this stimulated many clinical workers to investigate the possible application of specific chemotherapy to cases of infected wounds.

Consequently, Leitch[10] used brilliant green 1 : 1,000 in aqueous solution and in normal saline solution as an antiseptic for infected wounds. He very soon observed that vital tissue under the action of the dye retained its normal appearance and color, whereas dead or necrotic tissue was deeply stained with the dyestuff. He also noted that the dye appeared to exert a more selective action upon staphylococci than upon streptococci.

A short while later, Short[11] reported upon the injection of a paste, composed of boric acid, French chalk, and brilliant green 1 : 200 in liquid petrolatum, into the sinuses of infected wounds with excellent results. They injected a 1 : 200 aqueous solution of brilliant green into the wound just before injecting the paste. They also maintained that there was no tissue destruction on the part of the dye and asserted that it was mildly stimulating to granulation tissue.

Browning, Gulbransen and Thornton[12] tested acriflavine, proflavine and brilliant green with especial reference to their suitability to wound therapy. They found in the test tube that brilliant green 1 : 2,000 aqueous solution is 500 times as toxic for a twenty-four hour broth culture of *Staphylococcus aureus* as is Dakin's solution, and it is 700 times as toxic as Dakin's solution for *B. coli*. Consequently, they used repeated irrigations of 1 : 2,000 aqueous solution of brilliant green to infected wounds and obtained excellent results. Furthermore, they found that it was absolutely innocuous to vital tissues and also that the dye is not reduced or hampered in any way in its activity by tissue juices and wound secretions. They did note, however, that blood serum in the test tube does reduce the activity of brilliant green in its toxicity for bacteria.

Later on, Browning[13] ascertained that brilliant green will kill gram-positive cocci in dilutions of 1 :100,000. He also says that in dilutions of 1:500, it stimulates phagocytosis. Similar results were obtained by Webb[14] and by Ligat,[15] both of whom used a 1:1,000 solution as irrigations in infected wounds. J. E. Adams,[16] in the treatment of erysipelas, used a 5 per cent. aqueous solution of brilliant green, picric acid and tincture of iodin in separate, yet comparable, cases, and concluded that the dye gave the most satisfactory results. It was painted upon the affected skin surface once or twice a day, according to the severity of the infection. No dressing was necessary, save to protect the clothing.

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[10] Leitch, A.: Brilliant Green as an Antiseptic, Brit. M. J. **1**:236 (Feb. 1) 1916.

[11] Short, A. R.; Arklé, J. S., and King, C.: Brilliant Green Paste in Wounds, Brit. M. J. **2**:506 (Oct. 20) 1917.

[12] Browning, C. H.; Gulbransen, R., and Thornton, L. H. D.: Acriflavine, Proflavine and Brilliant Green with Reference to Suitability to Wound Therapy, Brit. M. J. **2**:70, 1917.

[13] Browning, C. H., et al.: Brilliant Green in Infected Wounds, Brit. M. J. **1**:73 (Jan. 20) 1917.

J. W. Churchman[4] used dilutions of gentian violet in the treatment of purulent arthritis. He first tapped the joint to ascertain the type of infection. The joint was then lavaged and stained as outlined in a previous article.[17] He obtained a cure with good functional results in these types of joint infection: *Staphylococcus aureus*, two cases; pneumococcus, one case, and, gonococcus, four cases.

One lavage and staining was sufficient to procure an uneventful recovery in each case.

He[18] also treated several infected wounds of the skin and serous membranes with excellent results. He suggested the use of solutions of dye injected into the gums in the treatment of infections deeper in the tissues as well as superficial gingivitis. He prefers the term "bacteriostasis" to "bactericidal" in speaking of the action of dyes upon organisms.

Mackie[19] endeavored to clean up diphtheria carriers by using a spray of an aqueous solution of brilliant green (1:250 or 1:500) into the mouth and pharynx. He sprayed, three times daily, for one week, and asserts that he obtained negative cultures after such a spray.

This work was repeated by Kolmer,[20] who also used a 1:250 solution of brilliant green. He found that the disappearance of the diphtheria bacilli was merely temporary. He did not note permanent disappearance of the organisms.

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14. Webb, C. H.: The Value of Brilliant Green as an Antiseptic, Brit. M. J. **1**:870 (June 30) 1917.

15. Ligat, D.: Brilliant Green and Flavine in Infected Wounds, Brit. M. J. **1**:78 (Jan. 20) 1917.

16. Adams, J. E.: A Note on the Treatment of Cutaneous Erysipelas with Brilliant Green, Brit. M. J. **2**:779 (Nov. 20) 1920.

17. Churchman, J. W.: The Treatment of Acute Infections of the Joint by Lavage and Direct Medication, J. A. M. A. **70**:1047 (April 13) 1918.

18. Churchman, J. W.: Selective Bacteriostasis in the Treatment of Infections with Gentian Violet, J. A. M. A. **74**:145 (Jan. 17) 1920.

19. Mackie, H., cited by Browning, C. H.: Applied Bacteriology, Oxford University Press, London, 1918.

20. Kolmer, J. A.; Woody, S. S., and Yogle, E. M.: The Influence of Brilliant Green on the Diphtheria Bacillus, J. Infect. Dis. **26**:179 (Feb.) 1920.

Graham Smith,[21] who was investigating the value of acriflavine, proflavine (acridin dyes, derivatives of acridin, a base found in coal tar [22]) and crystal violet, found that the toxicity or inhibitory power depends to a great extent upon its alkalinity. Thus crystal violet inhibits *Staphylococcus aureus* as follows: 0.15 per cent. tenth normal hydrochloric acid, 1 : 1,500,000; 0.08 per cent. tenth normal sodium hydroxid, 1 : 3,500,000, and 0.58 per cent. tenth normal sodium hydroxid, 1 : 5,000,000.

From this it can be seen that the dye is three times as toxic for *Staphylococcus aureus* in alkaline solution as it is in acid solution, within certain limits.

Churchman has utilized chiefly gentian violet, which, as he says, is but 90 per cent. effective in its selectivity of gram-positive organisms, and only 10 per cent. effective against gram-negative organisms. He fails altogether to recognize the value of such dyes as brilliant green, whose sphere of selectivity is the gram-negative type of micro-organisms. Thus, in using a mixture of brilliant green and crystal violet one would have a therapeutic agent whose selectivity was not only 100 per cent. effective against gram-positive organisms, but against gram-negative ones as well.

TABLE 1.—COMPARISON OF ACTION OF GENTIAN VIOLET AND CRYSTAL VIOLET

Location	Hours	No. Cases	Sterile
Abdominal skin........	6	22	22
Peri-anal skin..........	3	20	Growth in two of four streaks in one case; growth in one of four streaks in two cases.

Browning [23] compared the action of gentian violet and crystal violet and found them to be practically identical in action. In sterilizing the skin, he used a mixture of 1 per cent. each of brilliant green and crystal violet in 50 per cent. alcoholic solution. He washed the skin off with the solution and applied a compress soaked with it. He obtained the results given in Table 1.

In order to ascertain what becomes of the dye in the animal body, much work has been done. Perhaps the most authoritative of which was done by Churchman.[4] He injected solutions of gentian violet into the marginal vein of a rabbit's ear, and one and one-half hours later, when blood was drawn from the ear, no dye could be demonstrated by its bacteriostatic power. This was not due to mere contact with the blood as he proves, nor was it due to oxidation on the part of the blood. Venous blood and arterial blood proved the same in their actions.

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[21] Graham-Smith, G. S.: Some Factors Influencing the Action of Dyes and Allied Compounds on Bacteria, J. Hygiene **18**:1, 1919.

[22] New and Nonofficial Remedies, J. A. M. A. **73**:1443 (Nov. 2) 1919.

[23] Browning, C. H.: Footnote 20, second reference. Browning, C. H., and Bonney, V.: Sterilization of the Skin and Other Surfaces with a Mixture of Crystal Violet and Brilliant Green, Brit. M. J. **1**:562 (May 18) 1918 and Footnote 4.

When the dye was taken up by epithelial cells it did not break down so rapidly—the change was much more gradual. There was no tissue destruction on the part of the dye when used in dilute solutions. At the same time, the dye persisted in the tissues unchanged for sufficient time to produce bacteriostasis before it was finally disposed of. This valuable property is not found in other bacteriostatic substances.

Strasznor[24] found that methylene blue was reduced by (—SH) sulfhydrid groups in the tissues. This activity on the part of the tissues is reduced by heating them, but is restored again by the addition of tissue containing (—SH) sulfhydrid groupings. It is entirely possible that the sulfhydrid grouping may be responsible for the reduction in vital tissue of members of the triphenylmethane derivatives as well.

BACTERIOSTATIC VALUE OF BRILLIANT GREEN AND CRYSTAL VIOLET

As a result of these findings we have undertaken to ascertain the bacteriostatic value of brilliant green and crystal violet when acting together in solution, and to standardize, if possible, the technic of application of such solutions.

In the following experiments Grübbler's dyes were used except where specially indicated.

We have repeated in part the experiments of Browning, by first swabbing the skin of the abdomen and about the anal orifice with a mixture of 1 per cent. brilliant green and 1 per cent. crystal violet in 50 per cent. alcohol. Cultures were made of a series of cases by scratching the skin with a sterile needle and stroking an agar plate. Four strokes were made in each case, the needle being flamed between strokes. Cultures were made of another series after painting the skin surface as above, but in addition, applying a compress soaked in the dye and later taking cultures in the manner just described (Table 2).

A third series of experiments was conducted thus: The skin was washed with soap and water and rinsed with 4 per cent. sodium bicarbonate solution. Following this a compress of aqueous solution of 1 per cent. each of brilliant green and crystal violet was applied. Cultures were made of areas so treated as heretofore described.

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[24] Strasznor, Walter: Die Reduzierenden Wirkungen des Gewebes, Biochem. Ztschr. **29**:295, 1910.

The time element in each case was five hours in the following series of cases, from which one would conclude that it is necessary to apply the compress about the anus, in order to obtain sterility in 100 per cent. of the cases (Table 3).

From the data given in Tables 2 and 3 it may be readily seen that aqueous solutions are just as satisfactory as alcoholic solutions when applied over longer periods of time.

Considerable success has been encountered in treating secondarily infected wounds of the skin and subcutaneous tissue. The first consideration is to insure free drainage and then to apply a compress soaked in aqueous solution of 1 per cent. each of brilliant green and crystal violet. This dressing should be changed two or three times a day depending upon the amount of drainage. Care should be taken to get the dye in contact with all of the wound surfaces.

The application of alcoholic solutions of mixed dye to chronic leg ulcers caused each one so treated to heal remarkably fast, whereas other cases, quite similar but treated otherwise, showed no sign of closing over.

TABLE 2.—COMPARATIVE RESULTS WHEN COMPRESSES WERE APPLIED AND WHEN NOT APPLIED

	No Compress		Compress Applied	
Location	No. Cases	Sterility	No. Cases	Sterility
Abdominal skin..............	10	6	16	16
Peri-anal skin...............	18	8	12	12

TABLE 3.—RESULTS OF APPLICATION OF COMPRESS ABOUT THE ANUS

Location	No. Cases	Sterile
Abdominal skin.............................	13	13
Peri-anal skin..............................	13	13

Similar results have been obtained in the treatment of chancroidal infections.

Mr. E. W., single, aged 53, had large varicose veins of both legs with an ulceration 3.5 cm. in diameter on the lower third of the right tibia. The ulcer has been present and draining for seven months without a sign of closing over. The patient, without stopping his regular work, was treated with daily applications of 1 per cent. brilliant green and 1 per cent. crystal violet in 50 per cent. alcohol for five days. At this time, the discharge had practically stopped and healthy granulations had formed at the base, epithelium growing in from the edges. The dye therapy was continued three times a week for two weeks more, when the ulcer had entirely healed.

The hydrogen ion concentration (p_H) of saliva varies greatly in different individuals, and also in the same individual from time to time This change may be brought about by changes in diet, appetite and general health, by psychic changes, and by a pathologic condition of the oral and gastric mucosa and teeth. Bloomfield[25] finds that the saliva from normal people varies between p_H 6.0 and p_H 7.3[26] and that 80 per cent. of the specimens fell between p_H 6.6 and p_H 7.1. Persons who were suffering from a variety of diseases had a slightly wider range (p_H 5.8 to p_H 7.5) than was found in the normal group.

I[27] likewise examined a series of specimens of saliva of normal people and found that the average was p_H 7.43.[26] This is fairly alkaline and is sufficient to enhance the value of crystal violet and brilliant green when acting in the oral cavity. It has been demonstrated in previous work that these dyestuffs are more effective in alkaline than in acid mediums.

For this reason, I recommended the use of a mouth wash of 4 per cent. sodium bicarbonate solution after carefully brushing the teeth with an alkaline tooth powder. Then a mixture of 1 per cent. each of brilliant green and crystal violet in 50 per cent. alcohol was applied to the surface of the gums. Three minutes later, cultures were taken from this area and proved sterile. Further observations showed that the alcohol alone did not account for the sterility obtained. Aqueous solutions of the dye used over a very limited period of time (2.5 minutes) did not give the same result, and it was concluded, in order to obtain the result sought in the shorter period of time, that the alcohol was necessary to carry the dye. No experiments were undertaken at that time to test the value of aqueous solutions over longer periods.

Infected maxillary sinuses are thoroughly irrigated with sodium bicarbonate solution by means of a trocar passed through the mesial wall below the inferior turbinate, and then injected with from 2 to 3 c.c. of aqueous solution of the mixed dye. The treatment may be repeated every day for several days, depending upon the severity of the case. In all, twenty patients have been treated in this manner, with excellent results in twelve. The cases of six of the remaining eight patients were complicated by further sinus infection and were only temporarily relieved, and two patients had open sinuses into the oral cavity, reinfection taking place very rapidly afterward.

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[25] Bloomfield, A. L., and Huck, J. G.: The Fate of Bacteria Introduced Into the Upper Air Passages. IV. The Reaction of Saliva, Bull. Johns Hopkins Hosp. **31**:118 (April) 1920.

[26] According to the colorimetric method of Clark, U. M., and Lubs, H. A. (The Colorimetric Determination of Hydrogen Ion Concentration and Its Application to Bacteriology, J. Bacteriol. **2**:191, 1917) and of MacLeod, J. J. R., (The Diagnosis of Acidosis, J. Lab. & Clin. M. **4**:315 [March] 1919).

[27] Berwick, C. C.: The Disinfection of the Oral Mucosa with Brilliant Green and Crystal Violet, J. Dental Res. **2**:21, 1920.

A large ulcer on the posterior pharyngeal wall had remained unchanged for a long time under treatment with ordinary drugs. After three applications of the dye in alcoholic solution at twenty-four hour intervals, granulations formed at the base of the ulcer and it healed in a very short time.

Acute pharyngitis and tonsillar ulcers are likewise benefited by frequent applications of alcoholic solution of mixed dyes.

The following is an outline of procedure for cleaning up gross infections in the mouth preparatory to surgical operations in the mouth, nasopharynx, larynx or trachea. First, the teeth are thoroughly cleansed by carefully brushing all surfaces with a hard, small, baby's tooth brush, using a mixture of equal parts of talc and sodium bicarbonate. Care must be taken not to brush the gums, for then tissue destruction will take place at a faster rate and the infection will be the more difficult to eradicate. This brushing should be done carefully and systematically, taking at least from ten to fifteen minutes to the entire mouth. The mouth is then washed out with an alkaline wash, either a 4 per cent. solution of sodium bicarbonate or the ordinary liquor alkalinus antisepticus solution. Following this, the gums and necks of the teeth should be painted with a 50 per cent. alcohol solution containing 1 per cent. each of brilliant green and crystal violet. Care should be taken to get the dye in between each tooth, and where it may enter the pyorrheal pockets about the necks of the teeth.[28]

This procedure should be carried out once the first day, twice on the next day, if possible, and once several hours preceding the operation.

This technic has been successfully carried out in many cases of oral and dental surgery, as well as in three cases of laryngectomy and several cleft palate operations.[29]

After operation in mouth cases, it is of benefit to swab the mouth and wound surface with alcoholic solutions of the dye at frequent intervals. This not only hastens the healing process but also serves to lessen the pain to a great extent, as illustrated by the case herewith reported.

Mr. J. A. P., aged 39, had osteomyelitis of the left mandible. The technic just described was carefully carried out before operation, and at operation the infected portion of the jaw was removed and drained. The wound was dressed daily with gauze packs soaked in 1 per cent. each of brilliant green and crystal violet (aqueous). In six days, there was no more discharge, and the packs were no longer inserted. In ten days the soft tissue had entirely healed over. Roentgenograms later revealed no local pathologic condition.

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[28] Considerable work is still being done to determine the effect of dyes on pyorrheal infections (J. Dental Research **2**:21, 1920) and we may state only that so far our observations tend to show that the dyes exert their action by merely temporarily lessening the symptoms caused by the infecting organisms. The tendency toward tissue destruction remains more or less unchanged.

[29] This technic has been adopted as the standard in the University of California Hospital, standing orders for cleaning up of gross infection in the oral cavity, preparatory to oral and neighboring operations.

For rectal cases, this method gives excellent results: Repeated soapsuds enemas are given until they return perfectly clear on the night previous to operation. The operative field, if about the anus, is shaved and painted with 1 per cent. brilliant green and 1 per cent. crystal violet in 50 per cent. alcoholic solution. Then about 20 c.c. of a 1 per cent. aqueous solution of brilliant green and crystal violet solution in aqueous solution is injected into the rectum and sterile dressings applied. On the morning of operation, the injection of aqueous dye into the rectum is repeated and the sterile dressings reapplied. When colostomy has been performed, the dye may be injected into the descending loop of the bowel before performing the Kraske or similar operations.

Postoperatively, the injection of from 10 to 20 c.c. of aqueous solution of dye into the rectum is of great benefit in the healing process, and the alleviation of pain is quite marked in many cases so treated.

Excellent results were obtained in four cases of resection of the rectum for carcinoma as well as in eight cases of hemorrhoidectomy.

Injection of the aqueous solution into rectal fistulas is of benefit in their excision, when they do not heal after repeated injections of dye into them. These fistulas may be easily located at the time of operation by the dark blue-violet color of their walls.

Ischiorectal abscesses, when irrigated with aqueous solutions of dye, heal more rapidly than with other substances. Of three cases so treated, the alleviation of pain was one of the marked features of the treatment, whether it was due to the cutting down of the infection or not could not be definitely determined.

Equally good results have been obtained in the treatment of unopened secondarily infected tuberculous abscesses in the ileopsoas muscle. The abscess cavity is aspirated of its contents by the use of an aspirating needle or small trocar. The cavity is then irrigated with a 4 per cent. solution of sodium bicarbonate until the fluid returns clear. A small amount of the soda solution is left in the cavity in order to maintain its alkalinity, and a small quantity of 1 per cent. aqueous solution of brilliant green and crystal violet is injected into it. The amount injected varies according to the size of the cavity. In a short time (from twelve to forty-eight hours), depending upon the nature of the infection and the size of the cavity, it will be found that the necrotic material is sterile. It then remains to permit the cavity to granulate in, with occasional lavages administered to remove whatever tissue débris remains in the cavity.

This method has been successful in six cases of *Staphylococcus albus* infection and one of *B. coli* infection of deep muscular tissues. In four cases of psoas abscess, the treatment was successful so far as it produced sterile cultures, but it was not possible to get at the source of the infection in the lumbar vertebrae. In three of these cases, the abscess cavity broke through the line of puncture and drained for a long time. The secretion not only was sterile upon ordinary enrichment mediums; but guinea-pig inoculation was negative for tubercle bacilli. These patients all died some time later from pulmonary involvement. No effort was made to ascertain the effect of the dye upon tubercle bacilli either clinically or in vitro, other than the above.

It has also been our observation that dead or necrotic tissue takes a deep stain and maintains it for a considerable period of time. This does not wash off with either alcohol or water to the extent that the dye does from vital tissue. Living tissue does not keep this heavy stain for so long a period of time, but the dye gradually becomes less intense until one can notice no stain remaining. This may be due, as Strasznor[24] has shown, to certain sulfhydrid groups inherent in the living cell. This activity is perhaps lost when the tissues undergo the process of decomposition, either by a catabolic or destructive action on the part of infective organisms.

ADVANTAGES AND DISADVANTAGES

The advantages of the use of dyes as antiseptics may be briefly listed thus: (1) specific selective action; (2) nontoxicity to vital tissues; (3) high alcohol and water solubility; (4) great penetration, and, (5) ease of application.

The possible objectionable feature of the dyes as antiseptics is the almost excessive staining quality. Considerable difficulty is experienced at first by those who use the dye, in trying to prevent staining of contiguous clothing and skin when not desired. With experience, however, one accustoms himself to its use, and the staining of other areas becomes less objectionable. This dye may be removed by immediate washing with alcohol, or acid alcohol, or by applications of, first, oxalic acid solution, and then water, repeated alternately until the stain is entirely removed. Hypochlorite solution is also of value in removing these stains. The same is true of the removal of dye from undyed cloth.

SUMMARY

There is a definite place for certain dyes in the treatment of wound infections. These dyes are extremely valuable on account of their selective bacteriostatic action, their low tissue toxicity and their ease of application.[30]

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[30] In addition to the references already given the following will be found of interest:

Holleman, A. F., and Walker, A. J.: A Textbook of Organic Chemistry, New York, John Wiley and Sons, Inc., 1915.

Churchman, J. W.: The Selective Action of Gentian Violet in Relation to Chemotherapy, Proc. Soc. Exper. Biol. & M. **18**:21, 1920.

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XI.

STUDIES IN ROOT-CANAL STERILIZATION[1]

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(From the Research Laboratories of the Department of Dentistry, and of the George Williams Hooper Foundation for Medical Research, University of California)

THE following report deals with an inquiry into the differences in histological structure of the organic matrix of the dentin and of the cementum, and constitutes the first part of a report upon the study of root-canal sterilization.

In preparing teeth for class-room work, it was noted that during the decalcification by acid, certain changes occur in the specimens which are not accounted for by the solution of the lime salts. From certain observed peculiarities, it was decided to determine to what extent this decalcification affected the organic portion of the tooth. The original plan of the experiment was to compare a ground section of a tooth with a section made from a decalcified specimen, for such a comparison would demonstrate the differences in the structure of the organic matrix from the structure of the ground section. Most authors agree upon the fact that the deposition of solid, inorganic portions of the tooth is made around the fine fibers of an organic matrix. For example, Noyes (1) states that the organic basis is formed first, and that "the inorganic salts are combined with it in a weak chemical union." Each tissue—enamel, dentin and cementum—is formed within its own matrix in a process which proceeds nearly simultaneously. Also, Mummery (2) and Von Ebner (3) have shown that this network in dentin is composed of a reticulum "of fine fibers of connective tissue, modified by calcification, and when that process is complete, entirely hidden by densely deposited lime salts." Finally, Hopewell-Smith (4) states that these fibers are identical to those found in bone, not only chemically, but also morphologically. He finds it impossible "to determine where the one begins and the other ends." Altho he does not state the fact, it is not unlikely that the author just quoted referred more particularly to the study of calcified, rather than decalcified sections. Experimental procedures to be described demonstrate that a separation between the matrix of the dentin and that of the cementum really occurs, and that definite histological differences exist between the two tissues.

METHOD OF STUDY

If a fully developed tooth is subjected to the action of dilute acids (nitric acid or hydrochloric acid), a solution of the inorganic material is effected. The residue which remains consists of the individual matrices which preserve the general outline of the tooth. The size, of course, is quite considerably reduced, but the difference between the crown and root portion is readily recognized.

The technic of decalcification, or of the removal of hard portions of teeth, has been developed and described by

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[1] Aided by grant from the California State Dental Society, and the San Francisco District Dental Society. Demonstrated before the California State Society, August 3, 1920.

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others in some detail. It consists, essentially, in hardening the tissue for twenty-four hours by means of Zenker's fluid, or by 10 per cent formalin dissolved in normal salt solution. The specimens are then washed, and gradually dehydrated in alcohol of varying concentrations (60%, 80%, 95% and absolute). In this treatment it has been noted that the usual time of twenty-four hours is insufficient for the thoro penetration into the portion of the tooth lining the pulp chamber, and it is necessary to lengthen this interval to forty-eight hours for each of the different strengths of alcohol used. Unless this is followed, the inner portion of the tooth does not "clear" well in xylol, and subsequent cutting results in mutilation of the tissue. For decalcification the specimen is hydrated by carrying it thru the different concentrations of alcohol from absolute to 60%, and finally washing in water. Decalcification is accomplished by immersing the teeth in a solution containing 1 gram phloroglucinol dissolved in 100 cc. of concentrated nitric acid, and this mixture diluted to 1 liter with a solution of 10 per cent nitric acid. It is advisable to use a relatively large volume of acid as compared with the volume of the tooth, and 150 cc. of the acid solution have been used to decalcify a tooth weighing approximately 2 grams. The temperature of the solution in some instances was raised to 37½° C., but it is preferable to choose a temperature of about 20° C., as, in my experience, less disintegration of the matrix takes place. Altho freshly extracted teeth were used to advantage, in some instances, in the experiments described, teeth which had been extracted for six months, but which had been kept immersed in a mixture of glycerin and water, were decalcified without apparent injury to the matrix.

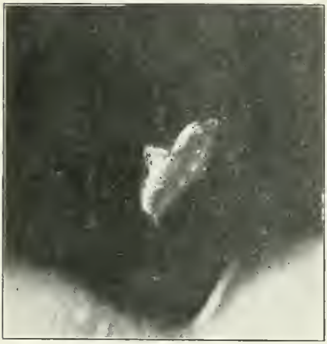

Fig. 1—First stage in the separation of organic matrix of dentin from cementum. The border of the tissue near the gingiva has been loosened, and is rolled back. Figs. 1-6, inclusive, show the macroscopic characteristics of the two matrices.

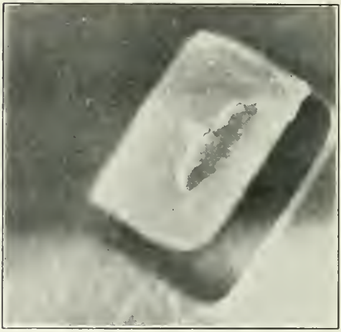

Fig. 2—The outer matrix has been loosened to a greater extent.

As a rule, teeth do not decalcify at the same rate of speed. It is, therefore, advisable to examine each specimen carefully at intervals of 12 hours. The decalcification, unless controlled, will eventually cause a disintegration of the organic tissue. To obviate this result, the tooth is removed from the acid and washed carefully in tap water until free from the re-agent. In my experience, the time of decalcification varies from 4 to 8 weeks, depending upon the size of the tooth, and, also to some extent, upon its age. Recently extracted teeth decalcify at a slightly more rapid rate.

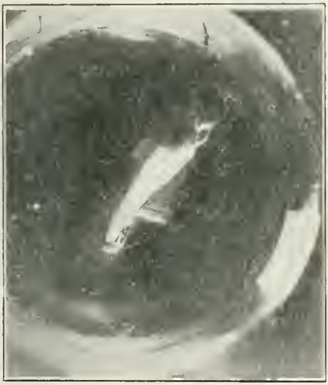

Fig. 3—The cementum matrix has been separated from the dentin at both borders, leaving a portion of the tissue adherent in the middle portion.

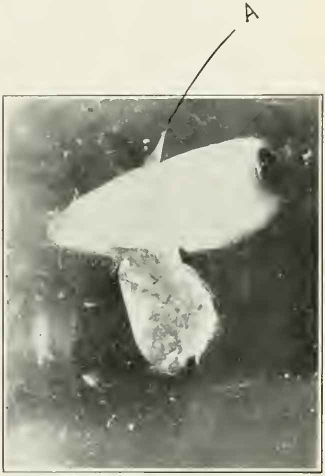

Fig. 4—An enlargement in which the general texture of the organic matrix of the cementum may be observed. A portion of the matrix is still adherent to the tooth as shown at "A".

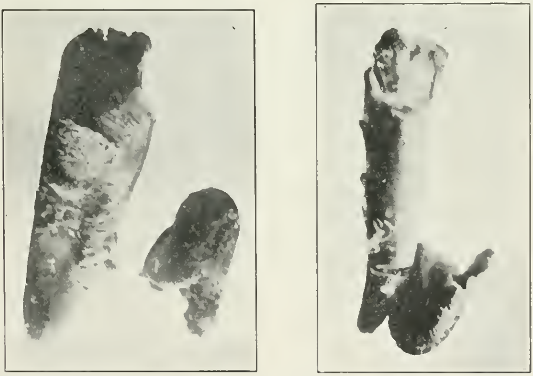

Fig. 5—The attachment of the outer tunic is severed, and the two tissues are shown in their relative positions.

THE RESULTS

The results of this study demonstrate three points:

(1) That the matrix of the dentin may be separated from the cementum.

(2) That the fibers of the dentin matrix run in a different direction from the fibers of the cementum matrix.

(3) That there is a slight difference in the size of the two matrices.

Figures 1 to 6 illustrate the progressive stages which may be followed in the removal of the outer tunic of the cementum from the inner tunic of the dentin. A carefully graduated pressing and pulling motion must be so applied that the direction of the force is toward the apex of the tooth. The ease of the removal depends entirely upon the degree of the decalcification of the specimen. If the process has been of too long duration, or if the acids used have been too strong, or if the temperature has not been controlled, the outer tunic becomes so friable that it breaks readily in the fingers. The matrix of the dentin, also, loses firmness, and fractures upon the slightest pressure. On the other hand, if the decalcification has not proceeded to a sufficient degree,

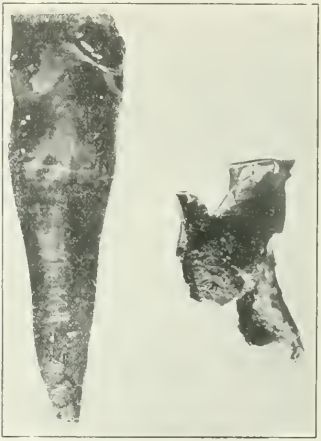

Fig. 6—The lingual aspect showing the complete separation of the two matrices.

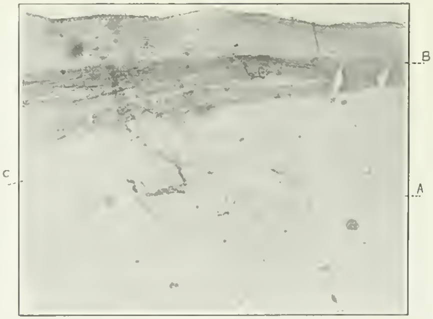

Fig. 7—A section from a decalcified tooth in which the matrix of the cementum is shown attached to that of the dentin. The difference in size of the fibers, as well as their relationship to each other, is shown at "A" and "B." Due to the great friability of the tissues, the section shown contains certain fibers in the field which have broken loose and which have assumed positions abnormal to the rest of the tissues. Such a condition is indicated at "C," the dark line of the fibers having an appearance of a "J." Figs. 7-10, inclusive, show the microscopic characteristics of the two matrices.

the calcium salts still remaining in the specimen make a separation of the two tissues very difficult.

The microscopic study of the tissues shows structural characteristics which are more pronounced in the sections than in the photographs. The illustrations, however, are sufficiently clear to indicate that there is a very marked difference in the relative position of the fibers of the dentin matrix and those of the cementum matrix. In the *dentin,* the direction of the fibers usually appears to emanate from a series of central points. They are analogous to the spokes of a wheel, except that the distance between the fibers does not increase as the fibers proceed from the center. In other words, the fibers parallel each other, and this parallelism is due, apparently, to a complicated anastomosis extending, presumably, from the pulp canal to the periphery of the dentin.

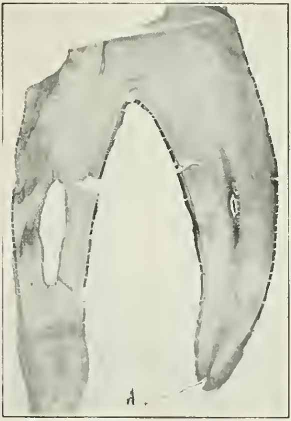

Fig. 8—A photomicrograph of a longitudinal section of a molar tooth showing location of apical foramen in distal root. The section is 8 mu in thickness. The difference between the cementum matrix and the dentin matrix is clearly indicated, especially in the region of the apex. Further up toward the hifurcation, the cementum matrix becomes so thin that it is hardly distinguishable in the microscopic section.

The fibers of the *cementum* present quite a different picture. In this case, they appear to run concentrically around the tooth, and in some sections, are at right angles to each other, as shown in Figure 7.

The difference in size of the fibers is not quite so apparent. The spaces between the fibers are greater, in many instances, in the cementum than in the dentin, and this gives a background on which the cementum fibers appear larger than those of the dentin. This difference in size is not constant, due to the fact that so far it has not been found possible to bring the different specimens to the same stage of decalcification. Consequently, the fine fibrous network is either partially disintegrated, which makes the spaces between the fibers larger, or else, the decalcification has not been entirely completed and a portion of the inorganic material still remains. In the latter event, the spaces are, in some instances,

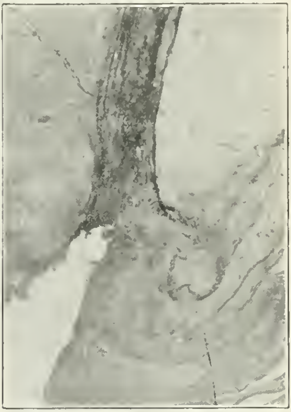

Fig. 8-A—Enlargement of specimen at "A" showing direction of cementum fibers and dentin fibers.

indistinguishable. The extent to which the fibrous network may be destroyed is shown in Figure 10.

It has not been possible to demonstrate in any of the sections a connection between the fibers of the dentin and the fibers of the cementum. They appear to be entirely different, not only in the direction in which they run, but, also, in their relative size. The fact that no connection between dentin and cementum exists, has already been demonstrated by Marshall (5) in experiments in which the tissues were stained by means of the vacuum principle. It was shown in these experiments that no connection exists between the ampulae of the dentin found in the Tomes layer and the lacunae of the cementum. This research upon the decalcified specimens would appear to substantiate that already accomplished by the author just quoted.

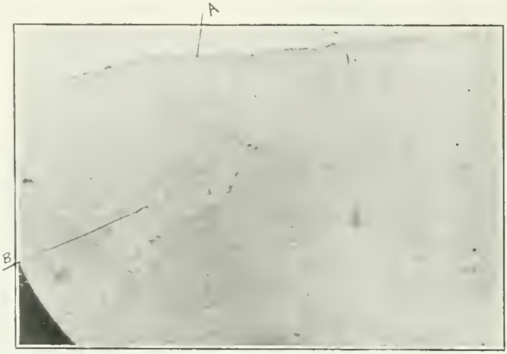

Fig. 9—**A longitudinal section of a dentin matrix from which the cementum matrix has been separated. The dento-cemental junction is indicated at "A". A partial disintegration of the matrix has occurred at "B" where individual fibers have been demonstrated.**

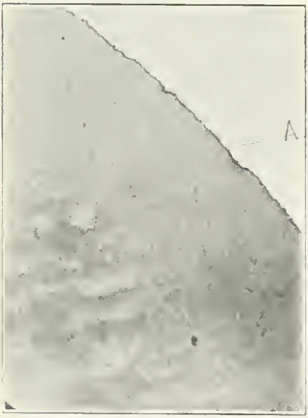

Fig. 9-A—**A decalcified but not disintegrated dentin matrix. The cementum of the matrix has been separated along the border indicated at "A". Note the radical direction of the fibers thruout the section.**

If the organic matrices of the dentin and cementum are connected, as has been supposed in the past, this connection would absolutely preclude the separation of the two tissues. Communication between the dentin and the cementum would be due to interlacing fibers which would provide channels of communication between the two tissues. Since a separation can be easily accomplished, it follows that the organic matrices of the dentin and of the cementum may be regarded as two distinct entities histologically different and with the exception of the apical foramen are without channels of communication.

The direct application of this research to root-canal technic and to the problem of sterilizing dentin is to be made the subject of a separate article.

Conclusions

A method is described which demonstrates new facts of histological interest. First of importance is the description of the separation of the organic matrix dentin and cementum. The technic followed in this work is exacting if a clear separation of the two tissues is sought. A macroscopic and microscopic study of the structures so treated indicates that this division of the two matrices would not be possible if any interlacing fibers existed between the dentin and the cementum.

The fibers of the dentin are characteristic in that they are radial in arrangement and those of the cementum are concentric.

In addition to this dissimilarity of arrangement, there is also a slight difference in the size of the fibers. Those of the cementum are larger than those of the dentin. This difference is partly dependent upon the degree of disintegration of the specimen incident to the decalcification. If the process is carried too far, the solution of the inorganic portion of the tooth is followed by a partial disintegration of the organic part. As a result, concordant results are difficult to obtain in specimens which have not been carefully controlled.

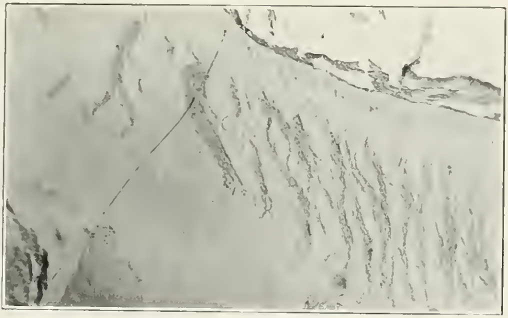

Fig. 10—Longitudinal section of partly disintegrated dentin matrix. The individual fibers may be seen broken away from the tissue.

I feel very much indebted to Miss R. Carson, Miss V. Litch and Mr. C. O. Tufts for assistance in preparing and photographing the material with which this paper is illustrated.

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STUDIES IN ROOT-CANAL STERILIZATION. II[1]

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IN A PREVIOUS paper (1), it has been reported that the decalcified organic matrix of the cementum and the organic matrix of the dentin may be separated in the same specimen by a definite laboratory procedure. This separation is possible on account of the fact that no interlacing fibers between the two tissues can be demonstrated. If such fibers exist, they would serve to bind these two structures together and would also provide a means of connection or communication between them. In the tissue so separated there is an observable difference not only in the arrangement of the fibers but also in their relative size.

Since these fundamental structures of the dentin and of the cementum do not appear to be united it becomes of interest to determine whether or not a connection can be demonstrated in the specimen which has not been decalcified. Such a condition is conceivable, and in fact has been reported by Howe (2). The results which he discusses are interesting only in that they afford a comparison of different laboratory procedures.

Two methods have thus far been used to settle this mooted question, namely, the so-called vacuum method of Marshall (3) and the ordinary method of staining, as reported by Howe. Judging from the results obtained by each of these methods, it is apparent that altho the former method is not entirely free from criticism, it is to be preferred over the latter for the reason that the technic of staining the specimen is more exact and is subject to more definite control. With the immersion method the dye penetrates both cementum and dentin. As a result there is a greater opportunity for misinterpretation of microscopic evidence than is the case when the vacuum principle is employed. By subjecting the specimen to vacuum staining it was shown that the penetration of the stain does not pass the dento-cemental junction.

Is it possible that these principles have some value from the practical aspect? This report describes the observations made on the extent to which solutions penetrate thru the walls of the root-canal into the dentin in conditions similar to those found in clinical practice. The depth of this penetration may be conveniently measured by colored solutions.

THE LABORATORY METHOD

Various dyestuffs may be employed for the purpose above outlined, but a mixture of crystal violet and brilliant green was found to be most suitable. Berwick (4) has described the sterilizing properties of an alcoholic solution of these two dyes. It occurred to me that this reagent, on account of its intense staining qualities, might have some advantages over others and, further, that on account of its antiseptic properties it might find an application in clinical practice.

A series of specimens were prepared

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[1] Aided by grants from the National Dental Association, the California State Dental Association and the San Francisco Dental Society. Demonstrated August 2, 1920, before the California State Dental Society, San Francisco.

in which the root-canals were cleansed and prepared for filling in a manner analogous to that employed under clinical conditions. The canals were dried thoroly and then treated with a 2 per cent aqueous solution of the dye. The application of the dye was made by means of a cotton point introduced three or four times successively in the canal.

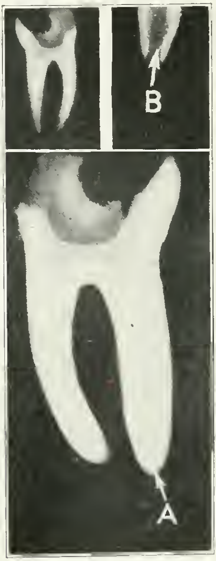

Fig. 1—Radiograph and enlargement of tooth treated by silver method. The white dots at the apex of one of the roots indicate the position of the accessory foramen and are caused by the deposited silver.

On the following day, the tooth was split thruout the length of the root-canal and examined microscopically. The splitting was accomplished by grinding two slits with a carborundum disk, in some cases lingually and labially, and in other cases mesially and distally. The direction of the slit followed the general direction of the long axis of the tooth, and paralleled, in many instances, the exact course of the root-canal. This was easily accomplished on account of the fact that as the disk approached the root-canal the stained dentin indicated the direction to be followed. Specimens were not cut thru with the disk in any case, for the grinding was stopped with the first appearance of a blue color. By inserting a knife blade, or a No. 20 Black chisel, the tooth was split in the direction of the two slits which had already been cut.

Sections prepared in the manner described have the advantage over those which are ground, either by hand or by machine, in that the material resulting from the cutting is of so coarse a nature that it can be easily removed from the tooth structure. Consequently the anatomical characteristics of the tissue are not obliterated by the fine dust and detritus which results from the preparation of ground sections. It has a disadvantage, however, in that the surface to be examined is sometimes very uneven, and the proper microscopic study of such a surface requires a considerable modification of focal length in studying the field.

Photomicrographs of the material examined show that in no case did the dye penetrate beyond the dento-cemental junction. The dye follows the course of the dentinal tubules to this point but does not penetrate into the cementum. Altho this connection between dentin and cementum cannot be demonstrated, some observers have pointed out that there is, nevertheless, the possibility of fluids passing thru these tissues. This, they explain, may be due to the fact that the structures are permeable and that osmosis occurs. Assuming that dentin and cementum possess properties in common with permeable membranes, this property exists only when both free surfaces are permitted to have access to fluids or gasses.

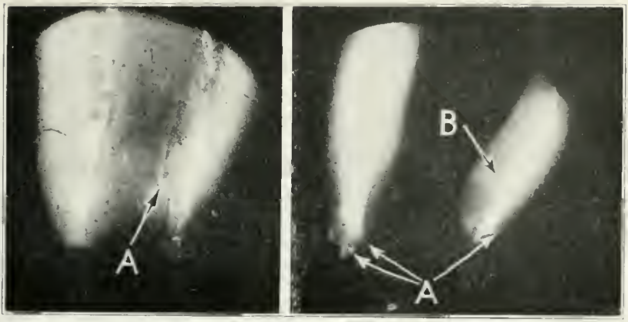

Fig. 2—Enlargement of radiograph showing penetration of colloidal silver in root-canal of molar. The outline of the root-canals is very definitely shown together with the position of accessory foramina as indicated at *A* and *B*.

By the term "free surface" I refer, on the one hand, to the outer surface of the cementum, or that surface lying next to the peridental membrane, and, on the other hand, to that portion of the dentin which forms the walls of the root-canal. If one side of a permeable membrane be so treated that the pores of the membrane become closed, the property of permeability is lost at once. It is by means of this property of permeability that the phenomenon of osmotic pressure depends. A substance or liquid may pass thru a permeable membrane under conditions which may be capable of control and of measurement, and the pressure which is exerted against the membrane by the passage of the liquid is termed "the osmotic pressure."

If dentin and cementum are permeable membranes, the first object to be accomplished in the filling of a root-canal is to treat one of the free surfaces in such a manner that this property of permeability is reduced to a minimum, if not actually lost. In addition to this, the application of antiseptic solutions, which would permeate or penetrate the dentin, would have an added value. This value would consist in the fact that when the

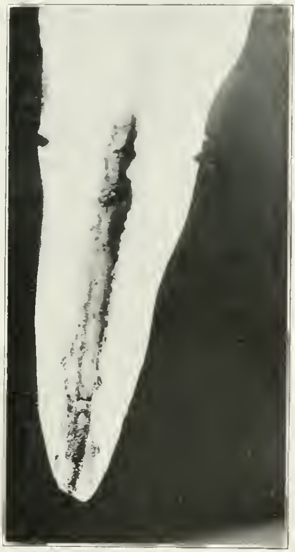

Fig. 3—Penetration of silver solution into walls of dentin

antiseptics are once applied to the dentin, and one of the free surfaces is varnished over, these antiseptics remain in the tissue. They remain permanently for the reason, first, that there is no connection between dentin and cementum whereby the fluids can be diluted and removed; and, second, if the root-canals be treated with certain substances, as, for instance, a solution of rosin dissolved in chloroform, the property of permeability, which we consider hypothetically as present, is reduced to a very considerable degree, if not entirely lost. No opportunity occurs under such conditions for the dissipation or dilution of these antiseptics. In those cases where multiple foramina exist, the possibility of sucessfully occluding the numerous openings is discussed in another place. At present the greatest success is attained by the application of the silver oxid treatment. Figure 1 shows the amount of penetration which has been obtained under clinical conditions. At *B* there is a portion of tissue which contains a greater deposit of silver than in the surrounding region. On inspection of the specimen it was found that there was a lateral foramen at this point which opened about one-third the distance from the apex to the bifurcation of the root. The silver had penetrated into this foramen and had deposited there, without the use of

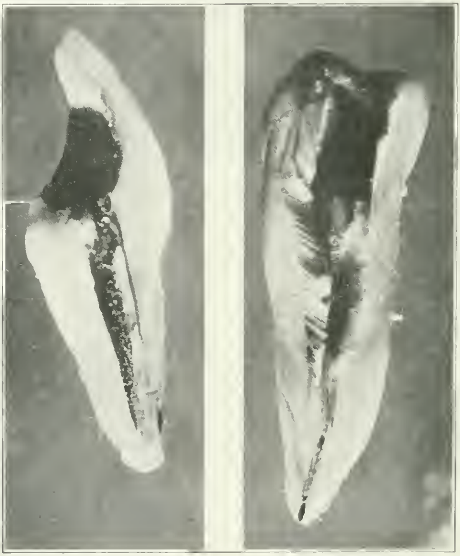

Fig. 4 Fig. 5

Figs. 4, 5—Fig. 4, incisor tooth, the crown of which was not properly insulated from action of crystal violet and brilliant green. Fig. 5, bicuspid tooth showing result of lack of insulation of the crown.

any instrumentation. In fact, instrumentation in a case of this sort is obviously impossible.

The almost universal success which the Howe treatment has attained is due to the fact that the precipitated silver may be regarded as a permanent antiseptic, and as such is capable of resisting the action of bacteria. The use of this solution, when correctly applied, permits a thoro penetration of the silver into the tubules of the dentin and such a penetration is permanent for the reason already stated. It is possible, however, to avoid the inflammation which sometimes follows the use of Howe's treatment, by substituting for the silver salt a solution of crystal violet and brilliant green. This solution penetrates with even greater facility than does the silver salt, and has, apparently, an equal antiseptic value. The extent to which the penetration of the dye occurs is indicated in Figures 4-8. In this particular case the specimens were treated in the laboratory in a manner somewhat similar to that which would be employed in treating the tooth in the clinic. Freshly extracted teeth were carefully cleansed, and the root canals dried by means of cotton points and heated wire. Following this a solution of crystal violet and brilliant green, 1 per cent each in 50 per cent alcohol, was applied to the canals three or four times successively by means of a cotton point. The dye was allowed to penetrate for a few moments, and then the teeth split in a manner similar to that described in a foregoing paragraph.

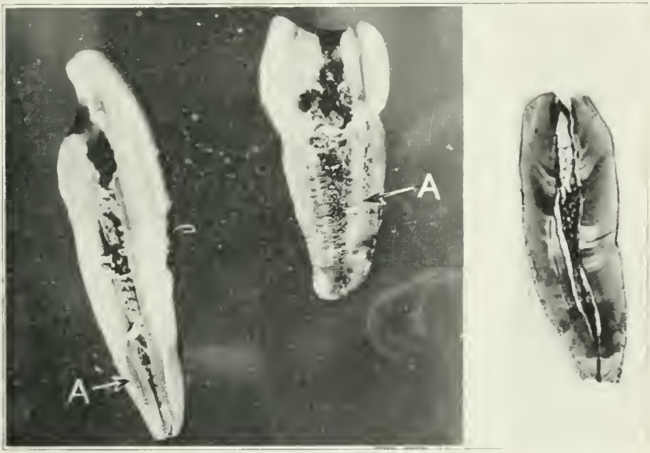

Fig. 6

Fig. 7

Figs. 6, 7.—Fig. 6, crowns of specimens protected by means of varnish and wax, extent of penetration of crystal violet and brilliant green in the root portion indicated at *A*; Fig. 7, penetration thruout tooth of crystal violet and brilliant green.

The dark area surrounding the root-canals is produced by the intense color of the dye. This penetration, it will be noted, has gone as far as the dento-cemental junction, but in no case has it passed thru this junction.

The same remarks apply to the sections showing the staining and penetration which may be expected from the use of the silver solution as described by Dr. Howe. As a consequence of these findings, it has been possible to develop a clinical procedure for the treatment of root-canals, which has been followed in the clinic at this institution for the past year with visible success. Cases have been observed clinically and bacteriologically, and an extended report covering this phase of the research is reserved for a subsequent article.

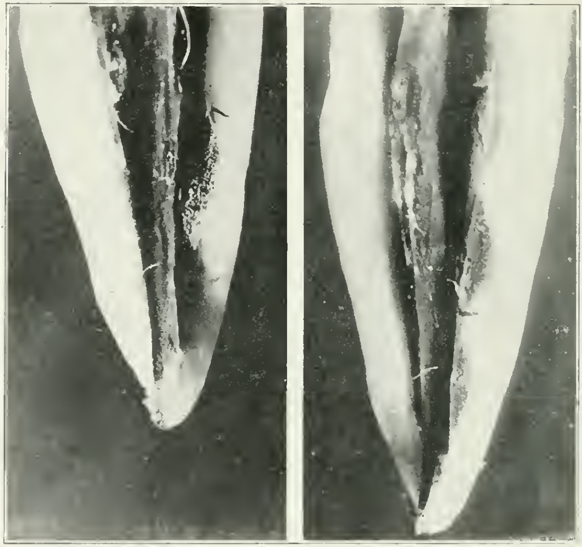

Fig. 8.—Enlargement of two incisors showing degree of penetration of crystal violet and brilliant green.

CLINICAL PROCEDURE

In the use of crystal violet and brilliant green it is necessary to observe the same precautions as those which are followed in the application of the silver solution. Particular note is to be made of the fact that treatment with this solution is governed by definite technic. The intense staining qualities of the dye render it objectionable when anterior teeth are to be treated. It is possible, however, to overcome this handicap if the crown of the tooth is varnished and waxed before the dye is applied. The solution should not be permitted to come into contact with any of the dentin surrounding the pulp chamber. In the case of anterior teeth, it is sometimes advisable to carry the wax down nearly into the gingival third of the root-canal. This is especially true where there has been any recession of the gum line.

The treatment, then, consists of packing the opening of the root-canal with three or four cotton points, and then varnishing the pulp chamber thoroly, as well as other portions of the crown. Following this, melted wax should be used as an additional protection against the solution, the wax being applied by means of the hot spatula. When the wax has cooled sufficiently, the plugs which are in the root-canal are removed and the root is ready for the application of the dye.

The tooth must be so prepared that there is no difficulty in carrying a cotton point from the dappen glass to the canal itself. After two or three successive applications of the dye, five to ten minutes should elapse to allow the material to thoroly penetrate into the dentin surrounding the canal.

Finally, the excess of the dye is removed by dried cotton points, and the canal thoroly desiccated by means of successive applications of alcohol or acetone and finally by a warmed wire.

The next step is to varnish the walls of the root-canal completely, using a 10 per cent solution of rosin and chloroform, as has been outlined by Callahan and others. The careful and thoro application of this liquid is of primary importance. It is essential that the canal be dried before any of the rosin solution is applied. Otherwise, the effect sought for, namely, the varnishing of the walls of the canal, will meet with no success. Finally, the usual solution of chloropercha is worked into the canal, and the *measured* gutta-percha point carried into place.

The shrinkage of the solution of gutta-percha is partly overcome by the rosin preparation; for the rosin appears to act as a binder to the guttá-percha. Teeth so treated have been examined by means of the radiograph, as well as by means of bacteriological tests, and while success has not attended every case, the results are promising. The reports of the bacteriological findings will be made the subject of a separate article.

Conclusions

1. This paper correlates the laboratory study of root-canal sterilization with clinical practice. The degree of penetration of antiseptics into dentin is illustrated by employing solutions of crystal violet and brilliant green.

2. In many cases the dye penetrated thru all of the dentin substance to the dento-cemental junction, but in no case was it possible to demonstrate the penetration into the cementum.

3. In comparing the action of these dyes with that of the Howe solution (silver nitrate treatment) it is shown that the degree of penetration is apparently equal. The discoloration of the tooth structure may be controlled by varnishing and waxing the coronal portion of the tooth.

4. The possibility of osmosis occurring between the tooth substance and the surrounding tissues is discussed. Since there is no demonstrable connection between the dentin and the cementum, except thru the apical foramen, antiseptics properly applied to the walls of the root-canal remain in the tissue. Finally a technic is described for obtaining permanent sterilization of the dentin.

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THE BACTERIOLOGY OF PERIDENTAL TISSUES RADIOGRAPHICALLY SUGGESTING INFECTION *

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From the George William Hooper Foundation for Medical Research and the University of California Hospital, San Francisco, Calif.

The medical and dental literature for a number of years has been filled with reports and bacteriologic data concerning chronic alveolar infections. A critical review of the wealth of material reveals one oustanding feature, namely, that no single report is based on a uniform and reliable bacteriologic technic. This applies especially to the method of obtaining cultures. Moreover, the reports indicate a lack of standardized operative technic and some form of statement which would prove that carefully conceived series of controls have been carried out. It is not unlikely that the cultures obtained and described at some length in certain publications were merely contaminations from the oral flora.

For this reason I have undertaken to investigate the bacteriology of chronic alveolar infections as evidenced by shadow changes in the radiogram; to determine the relationship of the cultures obtained to the bacteria of the oral cavity; to ascertain, if possible, the relationship of chronic alveolar infections to systemic disease.

Method of Study.—In a previous article I[1] reported a method of sterilizing the oral mucosa which proved satisfactory not only from the laboratory point of view, but also from the clinical aspect. The cultures were made from tissues dissected from jaws showing definite radiographic changes. The material was obtained as follows: The teeth were first brushed with an alkaline tooth powder and then the mouth was rinsed with an alkaline lotion. The alkaline reaction of the tooth preparation intensifies the bacteriostatic action of the dye. The gums and mucous membrane of the buccal surfaces were painted with a solution containing 1% each of brilliant green and crystal violet, dissolved in 50% alcohol. Churchman has shown that it is possible to inhibit the growth of gram-positive cocci in dilution of gentian violet up to 1 : 5,000,000 in alkaline solution. Hence, in every instance care was taken to avoid a transfer of dye into the culture medium with

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* Aided by a grant from the San Francisco Society for Dental Research.

[1] Jour. Dental Research, 1920, 2, p. 21.

the swab. The solution was applied after packing the area off with sterile gauze or cotton rolls. The dye was allowed to penetrate the tissue two and one half minutes. The gum was then incised and a flap of gum and periostaeum laid back with a periosteal elevator. The buccal plate was then entered with a chisel and with a clean chisel or rongeur forceps a piece of tissue at least 4-5 mm. in diameter was removed aseptically. This was then placed in a culture tube containing 1% glucose-veal-broth and taken immediately to the laboratory. The tissue was macerated with sterile sand broth according to the method described by Rosenow. The tissue pulp was enriched in glucose-blood agar and in glucose-veal-broth P_H 7.4, to which were added 10% defibrinated blood. The tube in which the tissue was taken to the laboratory was also incubated as a control.

TABLE 1

General Summary

Type of Local Lesion	Hemolytic Strepto-cocci		Non-Hemo-lytic Strepto-cocci	Staph-ylo-coccus, Pure	Strepto-cocci With Staphyl-ococcus	Other Organ-isms	Sterile	Total Number Culti-vated	Age of Patients, Years
	Pure	Mixed							
Sclerosis.......	..	..	2	..	1	..	..	3	25-43
Necrosis........	1	1	8	5	14	..	3	32	32-65
Granuloma....	..	1	8	2	4	..	..	15	18-71
Cyst...........	..	..	..	..	3	2 unclassified anaerobes	4	9	38-64
Pericementitis*	..	..	4	1	5	..	2	12	24-62
Total......	1	2	22	8	27	2	9	71	

* I have used the term pericementitis to cover the terms alveolitis and periodontitis as used by McCormack.

Swabs were made from the surface of the gum after the application of the dye just before the surface was incised. They were washed out in glucose-blood-agar and the latter poured into plates. The washed swab was placed in glucose-veal-broth and incubated as usual. Invariably these control cultures were found to be sterile after 48 hours of incubation. Occasionally control cultures were also made of areas appearing normal in the radiogram.

The bacteria isolated by the method described in the foregoing were identified, and, as far as the streptococci were concerned, they were classified according to the method of Holman.[2] Later, with the appearance of Brown's monograph,[3] they were differentiated first by hemolysis and then with 1% mannite, lactose, salicin, raffinose, inulin and sucrose, in beef serum with Andrade's indicator.

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[2] Jour. Med. Research, 1916, 24, p. 377.

[3] Monographs, Rockefeller Institute for Medical Research, 1919.

Results.—The cultures were obtained from jaws which demonstrated radiographically certain distinct changes. These have been illustrated by McCormack,[4] and in this paper the tentative classification given by him has been followed. It should be said in this connection that the bacteriologic study was primarily undertaken to elucidate definitely the meaning of some of the radiographic changes demonstrated and discussed by McCormack. It is quite evident that the bacteriologic data failed on some occasions to confirm the original radiographic interpretation.

TABLE 2

CASES WITH SYSTEMIC DISTURBANCES

Case	Age	Disease	Number of Teeth Extracted	Postoperative Condition	Cultures Obtained
1	60	Atrophic arthritis	1	Not improved after 24 months	Hemolytic streptococcus
2	62	Neuritis	2	Improved after 25 months	Staph. albus Strep. salivarius
3	27	Hypertrophic arthritis	5	Very slightly improved after 22 months	Staph. albus Strep. nonhemolyticus 1
4	60	Arthritis deformans	6	Not improved after 22 months	Staph. albus
5	33	Neuritis	10	Very slightly improved after 23 months	Streptococcus alpha 2.1 Streptococcus beta 8.5
6	40	Occipital headaches	11	Not improved after 24 months	Staph. albus Strep. salivarius
7	62	Atrophic arthritis	2	Not improved after 20 months	Staph. albus Streptococcus alpha 1.1 Streptococcus beta 2.1
8	45	"Run-down"	2	Improved after 23 months	Staph. albus Streptococcus alpha 4.5
9	32	Acute arthritis	7	Worse after 24 months	Staph. albus Streptococcus alpha 2.1
10	37	Hypertrophic arthritis	2	Not improved after 16 months	Staph. albus Streptococcus alpha 1.1

Table 1 shows the results of our bacteriologic study classified according to the radiographic and pathologic picture. In all there were 71 cultures taken. Of the total number of cultures obtained, we find only 3 hemolytic streptococci and of these 2 were found in large necrotic areas whereas 1 was isolated from a granuloma. At the same time we note that there are in all 22 pure cultures of nonhemolytic streptococci, and 8 pure cultures of staphylococci. A large number of our cultures showed that the infection was a mixed one and that often there were 2 or more different strains of streptococci in the material used for culture.

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[4] Jour. Dental Research, 1920, 2, p. 467.

Two of the organisms we obtained from a large cyst were anaerobes and further classification of these bacteria was not possible. Originally the organisms were obligatory anaerobes but with replanting they became facultative anaerobes.

Nine specimens of tissue taken for culture remained sterile, 3 of these coming from necrotic areas, 4 from cysts and 2 from areas of pericementitis.

In addition to this group, 3 other specimens showing apparently normal bone regeneration following extraction were cultivated. All gave sterile cultures.

There were other cases besides those operated on for the removal of local changes, which deserve more detailed analysis (table 2).

TABLE 3

DATA CLASSIFIED ACCORDING TO THE TOOTH INVOLVED IN THE INFECTION

Tooth		Streptococcus	Staphylococcus	Mixed Infection	Sterile	Total
Bicuspid..............	Upper	6	4	8	3	21
	Lower	2	2	2	1	7
First molar...........	Upper	4	..	4	1	9
	Lower	6	..	2	..	8
Second molar.........	Upper	..	..	4	2	6
	Lower	1	1	..	..	2
Cuspid................	Upper	1	1	1	1	4
	Lower	..	..	..	..	0
Incisor................	Upper	..	1	1	..	2
	Lower	..	..	..	..	0
Third molar...........	Upper	1	..	..	..	1
	Lower	..	..	1	1	2

The cases shown in table *2* are those that had been thoroughly examined by competent physicians, and primary causes as well as sources of infection other than dental were eliminated. After removal of the dental infection, a sufficient period of time was given in each case for the symptoms to improve or the changes to decrease. The shortest period of time allotted was 16 months and the longest 25 months.

Only two patients improved definitely, whereas two improved slightly, one was made worse and the rest were unaffected. An examination of the cultures from these cases showed that there were three hemolytic streptococci, one in pure culture and two mixed with other organisms; one pure culture of staphylococcus was obtained in a particularly bad case of arthritis deformans which was not improved; the predominating type of organism here also is the nonhemolytic streptococcus.

Table 3 shows that the upper bicuspid area gave over 33% of the cultures and that the upper and lower bicuspid areas together gave 45% of the total.

The reason for this predominance of bicuspid involvement is perhaps evident when one considers the great variation in the position, size and shape of the pulp chamber and the pulp caval of the bicuspids, especially the superior. Hopewell-Smith [5] and Marshall [6] give the variation from the normal at about 50% in regard to the upper bicuspid, whereas the variation from the normal of the other teeth is much less.

There appears to be no existing relationship between the type of infecting organism and the tooth concerned.

COMMENTS

This investigation shows that 9 cases of 71 gave sterile cultures. Three of these were from large necrotic areas, four from cysts of the jaw, and two from alveoli which showed definite thickening in the radiogram and in the pathologic examination. This definitely proves that cultures can be taken in the buccal cavity uncontaminated by the oral flora. It also shows that all changes in the jaw as evidenced by radiographic findings are not infected. Thoma [7] "believes that all teeth which give roentgen evidence of bone involvement are infected." Our evidence certainly does not bear out this statement. For over 10% of our cases proved sterile although there certainly was radiographic evidence of apparent boney change about the teeth in question.

The examination of the cultures we obtained shows that they correspond to a great extent to the cultures of organisms of the buccal flora (Meyer [8]). Arnold [9] also finds that the "hemolytic and nonhemolytic streptococci found in normal and pathological throats were of the same varieties, when classified according to Holman's sugar fermentation tests."

Under ordinary conditions these may be innocuous, but, due either to a temporary increase in virulence of the organism or to a local lowering of resistance, the bacteria may have gained a foothold on the tissue and there produced their local effects. The lesions produced by these organisms, when situated deep in the jaw, appear to clear up spontaneously in a few cases only, but appear to remain locally until removed by the dentist.

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[5] American Textbook of Operative Dentistry, 1920, p. 83.

[6] Operative Dentistry, 1921, p. 14.

[7] Boston Med. and Surg. Jour., 1921, 184, p. 434.

[8] Jour. Nat. Dental Society, 1917, 4, p. 966.

[9] Jour. Lab. and Clin. Med., 1921, 6, p. 312.

That the majority of the organisms situated here are capable of producing pathologic processes elsewhere in the body appears from our data at least to be improbable. However, further investigation is being carried out to ascertain this point.

The cases showing hemolytic streptococci are three in number, one in pure culture and two in mixed growths. The virulence of hemolytic strains of streptococci have long been said by a few to be greater than the majority of the other types of this organism. In examining table 2, which contains the group of cases showing the so-called systemic disturbances, we find that the predominating type of streptococcus is the nonhemolytic, and in 80% of these cases, it was found in mixed culture, either with another type of streptococcus or with staphylococci. In one of these cases a pure culture of staphylococcus was obtained. It is true, however, that all of the hemolytic cocci we obtained were found in this group of cultures. It is not unlikely that a larger series of cultures may show different results.

The eradication of these local dental infections, if they are related to processes elsewhere in the body, should produce some indication of reparatory change in the latter, provided, of course, that sufficient time be given and the damage done is not irreparable. Of the three cases furnishing hemolytic streptococci in their dental tissues, only one showed a slight improvement in the general condition.

These facts may possibly be explained as follows: The patients had other foci from which bacteria may have originated and which act as a source from which reinfection may take place. There are permanent changes in the body and even with the removal of the cause, the damage already done cannot be repaired. The dental focus may have no relation to the systemic disorders since time enough was allowed in each of the instances cited to allow the condition to improve following the removal of the infection by radical measures. However, careful physical examinations were made in each of the cases in table 2. Special care was exercised to search for foci of infection other than dental and to rule out any other pathologic condition likely to produce the condition under observation. In each of these instances the only definitely demonstrable anatomic changes which remained were in the mandible or maxilla.

It must be remembered that extensive hypertrophic changes in the osseous system cannot be greatly affected by the removal of the cause. The progress of the infection may be altered to some extent but without effect on the existing process.

From our series it appears that there is no definite relationship between dental infections and the symptoms complained of by the patient. The histories are not as complete as one desires and a larger series of cases is therefore being investigated along this particular line.

CONCLUSION

In a series of 71 cultures no type of organism was found to be characteristic for any radiographic change which had taken place in the maxilla or mandible. Contrary to expectation, it was noted that 10% of the cases were sterile. It is not possible to ascertain from the radiogram the existence of an infection in any case. The bacteria isolated from areas showing radiographic change correspond to a great extent to those found in the oral cavity. It is not possible to state from the results that there is any definite relationship existing between dental infection and systemic disorder in more than a small percentage of suspected cases.

Etiology of Acute Gangrenous Infections of Animals: A Discussion of Blackleg, Braxy, Malignant Edema and Whale Septicemia.

STUDIES ON PATHOGENIC ANAEROBES. I

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ETIOLOGY OF ACUTE GANGRENOUS INFECTIONS OF ANIMALS

INTRODUCTION

A piece of work on which I have been engaged, concerning the tissue-invading anaerobes, involved the collection of as many strains of such organisms as possible. At the suggestion of Dr. Karl F. Meyer, a fairly large number of strains of animal origin were collected and analyzed. It was thought that, besides being in itself of economic importance, the investigation of such a collection would be of assistance in the study of organisms causative of gas gangrene in man, for which branch of research the available strains of human origin were found inadequate. As these animal strains in turn threw new light on the subject of the infections of animals, it was thought appropriate to present under the above title certain data in connection with them. It also seemed desirable to make a rather extensive critical review of the literature of anaerobic animal infections.

There are many obscure points concerning the anaerobic invasions of animals. Human wound infections with their resulting cases of gas-gangrene have been studied extensively during the period of the war and they are found to be varied in their etiology and frequently polyspecific in their nature. The anaerobic organisms causative of human gas gangrene are usually placed in what I believe to be three important groups, which are considered by some workers (Weinberg and Séguin (b), the Anaerobe Committee) to be three species, namely, Vibrion septique Pasteur (the bacillus of Ghon and Sachs); B. oedematiens Weinberg and Séguin (closely related to but not identical with B. oedematis maligni II of Novy); and B. Welchii (B. phlegmones emphysematoseae Fraenkel, B. perfringens Veillon and Zuber). Rarely other invading organisms are found which are not to be identified with the above. I cite B. histolyticus, B. fallax and B. aerofoetidus Weinberg and Séguin, B. egens Stoddard, and have myself found a few such organisms that appear to be new. In the war literature there seem to be a host of faulty identifications, of associations of unrelated organisms, and of descriptions of mixed cultures. I shall confine myself in this paper to a discussion of the infections of animals.[1]

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[1] The bibliography of these diseases is sometimes difficult to quote in an orthodox fashion. Various authors of textbooks give long lists of articles consulted by them, and frequently in the account which follows such a bibliography no indication is given as to which of the many authors is responsible for the statements made in the text. A large number of references in early volumes of veterinary journals are difficult of access. One textbook gives an excellent account of braxy with no bibliography at all, while other important textbooks give references to a few but not to all of their authorities. In general, I have tried, in referring to an authority who quotes from another, to give the quoted statements verbatim; for the sake of clearness I have inserted the page number with the name of the author in the text. A complete alphabetical list of references will be found at the end of the paper.

I. A GENERAL DISCUSSION OF THE PRINCIPAL ORGANISMS INVOLVED IN THE ANAEROBIC INFECTIONS OF DOMESTIC ANIMALS

The differentiation of the disease called blackleg from that which is usually called malignant edema has been undertaken in all modern textbooks of veterinary pathology. The criteria used for such differentiation are usually seriously open to criticism, and in some cases are the cause of decades of misunderstanding of the subject. So serious have these misconceptions become that vibrion septique strains have been used as standards by which to identify B. Chauvoei. I cannot otherwise explain the statements of M. Nicolle, Cesari, and Raphael, who correlate the two organisms. The fact that the Pasteur Institute sent this laboratory a strain of the vibrion septique group, which was known as one of LeClainche and Vallée's classic blackleg strains, lends color to the suggestion that Nicolle, Cesari, and Raphael did not work with true strains of B. Chauvoei. Weinberg and Séguin come to the conclusion, through a review of the literature, that vibrion septique and B. Chauvoei are identical (b). I find it impossible to confound the organisms of these two types if they are in pure culture and if adequate facilities are at hand for their study.

It may be broadly stated that the anaerobic invaders of mammalian tissue form a large group, the members of which are not truly parasitic but may become so under diverse conditions. The species of the invaders are many, and they may be organized into groups which may best be likened to genera. Chief among these groups in incidence in animal infections is the vibrion septique group, which has a wide range of pathogenicity among animals. Second in incidence is the blackleg group, which has a narrower range of pathogenicity among domestic animals. Oedematiens group and Welch group infections may occur in animals, but they are comparatively rare. One should keep in mind the possibility of the occurrence of infection by organisms of other groups, and the question of invasion initiated by proteolytic organisms is to my mind entirely open. We are only on the threshold of the study of the anaerobic tissue invaders. Careful investigation of a large number of strains is necessary before definite generalizations concerning them are warranted. As far as my investigations go, I am prepared to define with some accuracy the principal groups of anaerobic invaders. Later researches may compel me to change somewhat the details of these definitions. Such definitions are here given for the vibrion septique and blackleg groups only.

Recently a bacteriologist declared to me in a private communication that a certain strain corresponded to the descriptions of the bacillus of blackleg which were "found in the literature." The culture proved to contain several anaerobic organisms of various types. On thinking the matter over in the light of the reading that I had been doing I could not blame him for his diagnosis. Perhaps this paper will serve to stimulate a more exact study of a sadly misunderstood group of bacteria. It should be said, and with emphasis, that the careful study of the organisms involved is of infinitely more value than any unstandardized pathologic observations or any epidemiologic ones. In many cases in which reports are given concerning blackleg infections or malignant edema infections it is impossible to tell which type of organism was present, whether both types were present, or whether an organism of another group may not have been present. No one is qualified to make a statement as to the etiologic factor in an anaerobic infection without bacteriologic investigation, and the literature contains so many confusing statements as to the bacterial entities involved that no one but the fortunate bacteriologist who has several strains to study and the time to study them is qualified to determine even the group affinities with certainty. When, however, the nature of the groups is understood, and when a technic is developed for their differentiation, one does not understand how the two could ever have been confused. They stand clear and separate as night and day, and mixtures of the two are quickly recognized.

I. CRITERIA OF DIFFERENTIATION EVOLVED DURING THE PRESENT RESEARCH

To save much confusion I shall here insert a definite statement as to the behavior in my hands of the two chief types of anaerobic animal invaders. I do this with due apology to others[2] who have adequately described these types, in order that the reader may understand definitely what my criteria of distinction are. I base my group identifications of the vibrion septique type on two strains of this type (VS and MP) sent to Miss Robertson and to Dr. Meyer by the Pasteur Institute. They are not specifically identical but are both referable to the group as I conceive it. Both are supposed to have been strains of Pasteur's. The blackleg group identification with B. Chauvoei is based on the description of Arloing, Cornevin, and Thomas, see p. 39.

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[2] Von Hibler (a and c) has a clear understanding of both groups. Markoff, Wulff, and several other workers have a definite understanding of blackleg. Ghon and Sachs, Weinberg and Seguin (b), and Robertson (a) have adequately described the vibrion septique type. Meyer (a and c) and McIntosh give the salient characters of both types, and lastly both types are described by the Medical Research Committee (Special Report No. 39).

(a) The Blackleg Group.—I should define the blackleg group[3] as follows:

1. Pathogenic Characters: Organisms producing a deeply hemorrhagic inflammation and necrosis in the muscles of the guinea-pig, with some sanguinolent infiltration, with little or no gas production, little interstitial regional edema, and only local injection of the peritoneum and rarely any injection of the small intestines. In guinea-pigs, dying of blackleg infection, bile frequently enters the small intestines in large quantities; the gallbladder is usually empty, the intestines do not contain an unusual amount of gas; the peritoneal surfaces are not excessively moist. The blackleg organisms are not highly pathogenic for laboratory animals when compared with the organisms of the vibrion septique group; some strains are in my hands pathogenic for rabbits.

2. Morphologic and Staining Characters: Bacilli that are usually gram-negative (carbol methyl violet Gram stain, see p. 19, b). In the vegetative form, in animal tissue and in meat medium, they usually stain uniformly and palely. In the orgont[4] form and in the sporulating form they may or may not stain clearly, depending on the amount of carbohydrate in the medium. In case they stain clearly they stain less intensely than do vegetative forms; in case the protoplasm is granular, the granules may stain strongly, taking the Gram stain; the remaining substrate is pale and usually gram-negative. The vegetative forms are comparatively slender, usually averaging in a 24-hour meat culture at least four times as long as wide. Actual measurements vary greatly. The vegetative and sporulating forms of most strains show little tendency to form filaments. I have never seen strains of this group which form filaments on the liver of the guinea-pig. The orgont forms are often far larger than the vegetative forms. They are extremely polymorphic, varying greatly within the group. Some are rods, some are rounded or pointed oval forms, some are lemon shaped, some are almost spherical. Spores form early in the development of a culture—in meat medium after approximately 20 hours' growth, and sporulating rods may usually be found at the site of inoculation in the guinea-pig. Spores are always oval, never spherical; they may vary greatly in their proportions and size in one strain, sometimes being long with parallel sides; they are usually situated subterminally in the bacillus (though terminal and median spores occur) and they frequently lie sidewise in the bacillus. This character is more usual for some strains than for others; it is not characteristic of blackleg alone. Rods containing two spores are occasionally seen.

3. Cultural Characters: Strictly anaerobic organisms generally of shy growth habit, which show no proteolytic action on meat, serum, or egg mediums, but which liquefy gelatin. They do not in my hands grow on fresh brom-cresol milk alone but grow well even in old milk with blood in it, forming in 2-4 days a soft clot which is torn by ascending gas and is usually fragmentable

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[3] A detailed classification of the species that are represented in my collection and make up these groups, as well as certain related groups, is in process of elaboration.

[4] Orgont, participial stem of ὀργάω 'swell', properly 'swell and teem with moisture'; used, for example, of fruit swelling as it ripens. Compare schizont from σχίζω 'split.' I would suggest orgont as an adjective descriptive of the organisms that have lost their bacillary form or size and have swollen, presumably preparatory to sporulation. "Orgont form" may be taken to include all swollen forms in which spores are not visible, whether the fate of such an individual is sporulation or dissolution or even division. "Orgont" may be used as a noun, like schizont. Rods which do not swell preparatory to sporulation may be said to sporulate without "orgont formation." Orgonts may or may not show protoplasmic disorganization.

by shaking. They split glucose readily, producing gas in meat medium and turning it pink. They are strictly nonproteolytic in the anaerobist's sense of the term. They are violently hemolytic; they autoagglutinate with extreme readiness; they are flagellate and motile (see p. 18, a).

Their deep colonies in liver agar are small, rather slow to develop, fundamentally lenticular in structure and are subject to variations according to species. The colonies rarely show tufted projections and do so only after two or three days' incubation. These projections are not loosely woolly but are clumpy and downy.

(b) The Vibrion Septique Group.—I should define the Vibrion septique group as follows:

1. Pathogenic Characters: Organisms producing a moderately marked serohemorrhagic inflammation of the muscles of the guinea-pig, marked interstitial infiltration, a moderate degree of serous subfacial regional edema and usually a large amount of gas; and producing almost invariably extensive injection of the serous membranes and of the intestines. In guinea-pigs dying of a vibrion septique infection the bile is freely discharged into the small intestines in large quantity, the gallbladder is usually empty, and the intestines contain a large amount of gas. The peritoneal surfaces are excessively moist.

2. Morphologic and Staining Characters. Bacilli usually gram-positive (carbol methyl violet Gram stain, see p. 19, b), though not intensely so, as are the organisms of Welch and Bifermentans type. In the vegetative form in animals and in meat medium they stain uniformly and rather deeply with anilin dyes. In the orgont form, and frequently in the sporulating form, they stain irregularly. In the animal body a localization of deeply staining protoplasm in one or both ends is frequent, and barred rods are common in certain strains. Granules of protoplasm are gram-positive and take stains deeply; the substrate may be very pale and may even lose a Gram stain. The vegetative forms are usually comparatively chunky in meat medium and also in the muscles of experimental animals. When growing in meat medium, forms are usual in the case of most strains, which on the average are not more than three times as long as thick. Filaments are occasional in animal muscle and in meat medium, they are usual in the serous cavities of animals and reach their greatest size on the surface of the liver of animals. Some strains do not form such filaments until some time after the death of the animal. The orgont forms are extremely polymorphic in shape in animal tissue and are sometimes so in serum mediums, and they are not abundant in meat medium, sporulation occurring in such medium without the formation of large orgonts. "Chunky oval forms" are the common orgonts found in animal tissue. In general the orgont forms are smaller than the corresponding forms in the blackleg group. Spores form early in the development of a culture—in meat medium after, roughly, 18 hours' incubation; they may or may not be present at the time of the death of the animal. Spores are oval, never spherical and never square ended. They vary somewhat in size but usually very little in the ratio of their width to their length. They may be terminal or median in their location in the bacillus; median spores are frequent. Spores are usually symmetrically placed in the rod; two strains (AS and BRH) in my possession form at times spores which are placed distinctly sidewise. The formation of two spores in one rod is exceedingly rare.

3. Cultural Characters: Strictly anaerobic organisms of comparatively energetic growth habit, which show no proteolytic action on meat, serum, or egg mediums, but liquefy gelatin. They produce, in milk, in 1-4 days, gas and a soft clot which, at an early stage, is fragmentable by shaking but later stiffens, and is torn by small bubbles of ascending gas. They split glucose readily producing gas in 18 hours in meat medium and turning it pink. They are not so violently hemolytic as members of the blackleg group. They occasionally auto-agglutinate in liver broth. They are motile in actively growing neutral or alkaline cultures. They reach the zenith of their growth in about 20 hours in most mediums, and after three days no longer proliferate.

Their deep colonies in liver agar are large, quick to develop, and woolly, showing rarely (strains WS and VS), and only early in liver peptone agar, evidences of a lenticular origin; they are subject to variation according to species.

2. DIFFERENTIATION OF THE ORGANISMS INVOLVED IN ANIMAL INFECTION: JUDGED CHIEFLY BY THEIR ACTION IN THE ANIMAL BODY

The discussion of the criteria which have been considered as of value in the determination of these groups brings us to the history of the subject.

(*a*) *Differentiation on the Grounds of Putrefactive Action.*—The early history of the vibrion septique and malignant-edema entities is one of the most interesting in bacteriology. Most authors find the descriptions of Pasteur and of Koch too fragmentary to be significant. This I believe to be a mistake. Though it is quite impossible to make any specific determinations on the grounds of the descriptions given by Pasteur and by Koch, it is quite possible to place their organisms approximately. We are, however, forced to pin our sources down to two classic and oft quoted descriptions.

Pasteur's description is significant. He inoculated into a guinea-pig material from a cow that had been dead for three days, presumably of anthrax, and into another, material from a horse that had been dead for one day, presumably of the same disease. We are told only that the disease was suspected and that Pasteur saw anthrax bacilli in the smears. The two guinea-pigs died, showing *désordres epouvantables:* the muscles of the abdomen and of the four legs were extremely "inflamed." Gas was present in the form of pockets, especially in the axillae (*"Ca et là, particulièrement aux aisselles, des poches de gaz"*). The liver and lungs were pale, the spleen normal but at times diffluent and the organisms were present in great numbers in the serosity of the abdomen and about the intestine. They were filamentous in the serous cavities, and regularly became septicemic in the guinea-pig before death. I interpret Pasteur's description to be that of an infection caused by an organism of the vibrion septique group as described on page 5, b. To be emphasized are: extreme inflammation of the muscles, gas production, formation of filaments, infestation of the abdominal serous membranes, septicemia. We are in no position to decide whether either of the two guinea-pigs that were infected from the cow and the horse, and such animals as were infected from them, died of a mixed infection or of a pure one. But surely the infection described by Pasteur was not of the type that shows great contamination by proteolytic organisms, because in such infections gas is not confined in bubbles in the axillae, but forms a large bubble in the destroyed tissue. One is forced to read into such a description as this the things that are not mentioned, as well as those that are. A proteolytic infection in which proteolytic organisms are at all abundant has such an odor of putrefaction that the abductor does not omit to state the presence of such an odor. That other animals which Pasteur inoculated with earth did have a mixed proteolytic infection is of course more than probable. Such infections he later refers to several times: "Un animal va mourir de la putridité septique qui nous occupe, car cette devrait être définie la putréfaction sur le vivant." And: "La septicémie ou putréfaction sur le vivant est-elle une maladie unique? Non; autant de vibrions, autant de septicémies diverses, benignes ou terribles . . ." which shows that Pasteur never pretended that one type of organism was responsible for these infections. This apparently contradicts Perret's statement that Pasteur identified his vibrion septique with the septicemia of Davaine which produced no macroscopic lesions.

It may be concluded, I believe, that Pasteur sometimes worked with mixed inoculum, wherefore he mentioned putrid infections, but that he at other times purified his inoculum by animal passage and that it is from such strains, of which two are included in my collection, that we now have the type of organism that we today recognize as the vibrion septique of Pasteur, which is a strictly nonproteolytic organism. The fact that the *vibrions septiques* of Pasteur, of which we have strains, cause lesions directly comparable with those described by Pasteur on page 793 makes this a type more definite. I do not mean to imply specific determination on the grounds of these old investigations, as I believe specific determinations to be at all times questionable when made from printed descriptions.

The Malignant Edema of Koch: To read the original article in which Koch proposes the name "malignes Oedem" (Zur Aetiologie des Milzbrandes) after spending months in the consideration of the work of his followers who have discussed the subject of "malignant edema" and its causative organisms: to read it in the light of familiar acquaintance with the organisms common in wound infection—is a most illuminating experience. Koch did better work on the subject of anaerobic infection in 1881, with no culture mediums that would grow his organisms, with no Gram stain, with little to guide him, than did any one else till Novy described his B. oedematis maligni II in 1894. The abyss of bacteriologic misunderstanding into which some of Koch's countrymen have today fallen (W. Kolle, Ritz, and Schlossberger, 1918) could all have been avoided by the perusal of Koch's article of 1881, and by the judicious use of laboratory animals. It lies in the nature of things that the malignant edema organisms encountered by Koch were not all the same. But the type described by him on pages 52-56 of "Zur Aetiologie des Milzbrandes" is a type that we know today, and it is not the type to which the vibrion septique of Pasteur belongs. The photomicrographs (table 8, figs. 43, 44, 45) are, however, probably taken from organisms which do belong to the group of the vibrion septique of Pasteur.

At the moment of the writing of his description of the lesions produced by him in experimental animals and termed "malignes Oedem," Koch must have been unfamiliar with the type of lesions described by Pasteur and referred to above. This I judge to be the case because Koch accuses Pasteur of having worked with mixed infections and gives a translation of Pasteur's necropsy findings on the guinea-pigs inoculated from the horse and the cow, allowing himself the liberty of translating "poches de gaz" as "von stinkendem Gas" (noted also by Ghon and Sachs [b. 666]), for which performance I cannot see any justification whatsoever. Koch describes mixed infections following the inoculation of dirt into experimental animals, with their gas and their thin pus-like fluid (Jauche), and he speaks of the peculiar dirty-red coloration of the muscles, which he, quite without justification, interprets to be the color that Pasteur described as due to "inflammation." This type of infection Koch states to be due to a highly complicated mixture of organisms. If one should take this mixture, says he, and inoculate it in any considerable bulk into a new animal, a similar infection will result. But if one should inoculate into a new animal a minute dose from the first animal, one will produce a slower infection, which is caused by a single species of organism. For this type of infection Koch proposed the name "malignes Oedem." It is, I believe, to this description that we must turn for our type of the malignant edema bacillus of Koch, and not to the photomicrographs mentioned above. The infection described is classic. Strange that it should have lain so long forgotten, while the name "malignes Oedem," and the later name B. oedematis maligni Koch, should have been battered to and fro from laboratory to journal, from journal to textbook, until all semblance of the original pathologic type and of the original organism was lost. Koch's description is as follows: The liquid which fills the subcutaneous connective tissue near the site of inoculation of the experimental animal (sp. ? probably the guinea-pig[5]) is not purulent, but is a pale reddish serum without odor and without gas formation. It contains only one kind of bacilli which in size and shape are almost exactly like anthrax bacilli. (They are a little slenderer and are not grouped in chains like anthrax bacilli [55]). Usually they are nonmotile and only occasionally does one see a movement on the part of an isolated rod. The internal organs of the animal show few changes. The spleen is usually enlarged and darkened, the lung is pale and grayish red. The presence of organisms in the blood varies. The bacilli are short and do not form chains until some time after the death of the animal. They are present in numbers on the serous lining of the abdomen even when no organisms are found in the blood. In mice the organisms regularly become septicemic, in other animals they do not always do so. There are also certain points that must be read into this description. No mention is made of changes in the muscle. If a true pure vibrion septique infection had been seen by Koch at the date of the writing of his description, he would most certainly have mentioned the muscle affection produced by that type of organism, which was described by Pasteur as "inflammation," and he would never have dared attack Pasteur so energetically as he did. Koch does not speak of the consistency of the edema present, he simply calls the disease seen by him "malignes Oedem." Neither does he mention injection of the serous membranes of the abdominal cavity; he insists that the internal organs show few changes; he describes no "désordres épouvantables." The description is so clearly given that one does not hesitate to assign Koch's organism to the same group[6] as B. oedematiens Weinberg and Séguin and B. oedematis maligni II. Novy. The oedematiens strains 139 and Domange of Weinberg and Séguin and strain AB of my own isolation (human gas gangrene) all produce in the guinea-pig lesions exactly like those described by Koch (I have not noted spleen changes); the organisms in my hands sometimes become septicemic and sometimes do not, while organisms of the vibrion septique group always become septicemic, at least in the guinea-pig (this character is, however, not a good one as it is based in my case on cultural determination and in Koch's case on microscopic observation); the organisms enumerated above do not form chains on the abdominal serous membranes of the guinea-pig; they are practically nonmotile, while vibrion septique strains are usually motile; they are large, and resemble somewhat roughly B. anthracis, which organisms of the vibrion septique type cannot well be said to do; most important of all, they do not produce marked muscle lesions or changes in the internal organs as do vibrion septique strains almost without exception; and they produce no gas in the connective tissue of the guinea-pig. I hold most positively that the malignant edema bacillus as described by Koch is nonproteolytic, because Koch states that the liquid near the site of inoculation is "ohne Gestank." This opinion I base on my own necropsy findings. Von Hibler's bacillus X in no way resembles Koch's organism; neither does the organism of Ghon and Sachs resemble it. Koch, so often stated to have identified his organism with the vibrion septique of Pasteur, merely says: "die Oedembacillen von ihm (Pasteur) vibrions septiques gennant." As we probably have today no strain called "malignant oedema of Koch," which produced the anatomic picture so well described by Koch, we are not in a position to identify Koch's bacillus specifically with any other organism. We have certainly no right to place it in the vibrion septique group until we have unquestioned strains of the vibrion septique group which produce the lesions described in Koch's classic delineation. But I think we are in a position to place it in the same general group to which B. oedematiens and B. Novyi belong. It must, however, be borne in mind that the term "malignes Oedem" was applied to a disease and not to an organism, that Koch and Gaffky used this name for most of their infections that were produced in animals by the inoculation of dirt, and that it is more than probable that among such infections the vibrion septique type was the most usual one. It is unreasonable for us to expect that Koch should differentiate these types. Let us remember when he worked, and under what handicaps. Let us not conclude that Pasteur differentiated his infections with any certainty. Let us remember his confusion of vibrion septique infections with rabies, and remember how few of the followers of Pasteur and of Koch have seen the light in the matter of differentiation of anaerobic infections.

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[5] As the guinea-pig is mentioned several times on subsequent pages in Koch's paper, and as Koch declares that this animal is extremely sensitive to malignant edema, it is probably safe to conclude that Koch saw the disease in that animal. Whether he saw it in rabbits we do not know, but rabbits are not mentioned in the neighboring text.

[6] By "group" I do not here mean organisms that would necessarily be placed in the same genus by the systematist, but organisms producing the same type of lesions in the animal body.

We have here one of those peculiar anachronisms that come about with the lapse of time. Pasteur surely was not clear in his mind as to pure infections and impure infections. He called his "vibrions septiques" putrefactive, but described a nonputrefactive type of infection, and we cannot decide just where his bases of criticism lay. But the strains which have come down to us under the name vibrion septique Pasteur were evidently pure ones and were kept pure in the Pasteur Institute, and we have them today as pure nonproteolytic cultures. Koch, on the other hand, was to my mind perfectly clear as to the difference between putrefactive and nonputrefactive infections; later, he probably isolated his organisms, cultivated them, and gave them to the world. Gaffky also had, for his time, a remarkably clear understanding of putrefactive mixtures and pure infections. To what extent the cultures which were given out were the type described by Koch or the type described by Pasteur, and whether they were of Gaffky's isolation, we cannot tell. The disease described by Gaffky is different from that described by Koch. On page 87 he describes an infection in the guinea-pig entirely similar to that described by Pasteur and he speaks of his organisms as "vibrion septiques."

Perhaps it was from one of these guinea-pigs of Gaffky's that the material came from which Koch's photomicrographs were taken. Gaffky also describes another disease of the general type with which we are dealing which was pathogenic for rabbits and mice, and not pathogenic for guinea-pigs. In discussing Koch's organism we are forced to leave Gaffky's organisms out of consideration: it will cause confusion enough to discuss two strains isolated by the same man, let alone those isolated by two men. We must consider that in whatever way we look at these descriptions of Koch's and of Gaffky's, there is an inconsistency between them. These "authentic" cultures of Koch's, if they were pure when given to bacteriologists at large, must quickly have become contaminated, as anaerobe cultures nearly always do in the hands of the aerobist "layman," but they kept their name, "malignes Oedem." And other workers "isolated" other organisms with similar putrefactive contaminations, which produced lesions similar to those produced by the now contaminated cultures of Koch, probably similar infections to those scornfully described by Koch as mixed. Later von Hibler may have found such cultures, at least he described carefully a proteolytic organism as B. oedematis maligni Koch (von Hibler's X) without the slightest scientific reason for so doing. Weinberg and Séguin (b, 23), and Fraenkel and Zeissler emphasize this point.

Many proteolytic organisms are capable of invading guinea-pig tissue in pure culture when administered in doses of 3 c c or more. This was the basis of Colin's protests against the technic of Pasteur in 1881. I do not wish to be understood as including such dosage in pathogenicity tests. One c c of a 24-hour meat culture is a large dose for a guinea-pig, and I do not employ larger ones.

Ghon and Sachs described an organism from a human case of gas gangrene which they identified culturally with vibrion septique Pasteur. They were unable culturally to compare their organism with the malignant edema bacillus of Koch, as they possessed no culture of Koch's organism. They then patriotically proceeded to call their organism B. oedematis maligni Koch, although the name vibrion septique Pasteur had preference. They did this on the ground of identifications made by German and French workers in the eighties. As Weinberg and Séguin suggest (b, 82), if vibrion septique did not seem appropriate as a name for their organism, surely B. oedematis maligni was no more appropriate, and a new name should have been chosen. The need for such a name was felt, for the organism of Ghon and Sachs was culturally different from the usual conception of B. oedematis maligni, and in pathogenicity it was somewhat different from the usual conception of vibrion septique Pasteur, neither of which organisms had been adequately described, so that later writers (Von Hibler, Meyer, Kitt, Köves, Schlemmer) who have identified organisms with the bacillus of Ghon and Sachs have called it by the name of the describers. These identifications have been numerous because the description given by Ghon and Sachs is detailed and elaborate. Such organisms have usually been defined as nonproteolytic, as forming chains on the liver of animals, and sometimes as nonpathogenic for rabbits. It is, in fact, in recent days, rare to find a record of an infection attributed to the bacillus of malignant edema of Koch, as described by von Hibler, which fact may be interpreted as due to the more careful cultural methods of recent workers.

The bibliography and history of the endless discussion concerning the above dispute is given by Weinberg and Séguin (b, Chapter II and p. 79). Ghon and Sachs give a detailed résumé of all cases of gas gangrene and of similar infections which had been recorded up to 1904.

Von Hibler (a, 88) is emphatic in his statement that the bacillus of Rauschbrand is nonproteolytic. He grouped all organisms found by him which formed woolly colonies, which grew in filaments on the liver of guinea-pigs, and which were nonproteolytic in their action on mediums, under the name "bacillus of Ghon and Sachs." That these strains of von Hibler's were not all of the same species, I am convinced. For example, strain 6 (pl. II, fig. 4) forms on the peritoneal wall of the guinea-pig, 4 hours after death, oval club-shaped forms. Strain 2, which I received in pure culture through the courtesy of Professor W. Frei of Zürich, does not form such club-shaped rods on the peritoneal wall of the guinea-pig, though I have seen 2 other strains of the vibrion septique group that do so in mice. Strain 5 (Kolle u. Wassermann IV. pl. XIX.1) does not form such clubs on the liver of the guinea-pig 6 hours after death. I am emphatically of the opinion that this type of character is specific. Von Hibler grouped strains that formed woolly colonies and that grew in filaments on the liver of animals but which were proteolytic in their action on mediums, under the name B. oedematis maligni Koch. Weinberg and Séguin (b, 24) seriously criticise von Hibler for this classification, saying that his identifications are incomplete, adding that von Hibler never isolated a strain from man that corresponded to this conception, all his human cases of "oedème malign" being attributable to the bacillus of Gohn and Sachs. Von Hibler isolated his strains of B. oedematis maligni from a mule, from garden earth that was inoculated into an animal, and from a cow that died of puerperal septicemia. Of his cases that were attributable to the bacillus of Ghon and Sachs, 5 were from man and 2 were from cattle. I do not understand why Weinberg and Séguin insist that an organism of this type should come from a human case in order to be genuine. I have, however, in a series of some 200 samples of pathologic material from animals and man, never encountered any strain which corresponds to the bacillus of malignant edema as described by von Hibler. That the strains studied by von Hibler were occasionally impure is witnessed in mute testimonial by two photomicrographs (a, pl. XI, figs. 2 and 16) which unquestionably portray mixed cultures.

Kitt (e, 287) classes as one species most of the anaerobic organisms that infect wounds, and he gives to this conception the name used for the organism of Koch, in its Latinized form, B. oedematis maligni. Of the agar cultures of this organism he states: "Das Gas ist geruchslos. (Von einigen Autoren wird angegeben, das stinkende Gase sich entwickelen; Mischkultur? Verwechslung mit anderen Kadaverbacillen?") He mentions here no proteolytic invaders. But in discussing Rauschbrand (e, 301) he declares that milk may be used to differentiate Rauschbrand from malignant edema, as the latter liquefies it and produces a foul odor (Smith, E. von Hibler). He describes a mode of obtaining B. oedematis maligni by inoculation of earth into mice or guinea-pigs, and holds that if such an infection is a mixed one, another inoculation will give a pure culture of the malignant edema organism. My experience with such infections has been very different from Kitt's. Earth contains many species of anaerobes that will infect mice and guinea-pigs, and mixed infections may occasionally persist as such after several passages, though if a true organism of the vibrion septique group is present, it usually outruns all others in the first or second passage. Von Hibler describes several species of invasive anaerobes that were isolated in this manner. Kitt mentions the fact that spontaneous malignant edema infections are often mixed, but he does not mention any anaerobes as being contaminating organisms.

Hutyra and Marek (33) say of B. oedematis maligni: "Milk is changed in a few days into a watery fluid with gas formation and the collection of a layer of fat on the surface (Smith)"; and they say (41) concerning the blackleg bacillus: "Milk is curdled only imperfectly."

Lehmann and Neumann (500) state that a putrid odor is indicative of malignant edema and absence of such odor is to be taken as indicative of Rauschbrand.

Bongert (198) declares the bacillus of malignant edema produces "höchst übelriechende Gase verschiedener Art."

Nocard and LeClainche (380) state that the Vibrion septique of Pasteur liquefies coagulated serum.

Grassberger and Schattenfroh (a and b) declare the Rauschbrand bacillus is an exquisite butyric acid bacillus (nonproteolytic), and the malignant-edema bacillus (vibrion septique) is a proteolytic organism. (See, however, p. 42).

Von Werdt (a and b) recognizes the existence of a "true bacillus of malignant edema of Koch" which is proteolytic and to the discussion of which he devotes a chapter: "Malignes Oedem." He also recognizes the existence of a group of organisms which he calls the organisms causative of "Gasbrand" and to which he devotes another chapter: "Gasbrand und seine Erreger." These organisms comprise: Welch-Fraenkel's bacillus, Ghon-Sach's bacillus I., Novy's bacillus, von Hibler's VI, VII, and XI, Ghon-Sach's bacillus II, Klein's B. enteritidis sporogenes, Klein's B. cadaveris sporogenes, Markoff's pseudo-rauschbrand organisms, the bacillus of malignant emphysema of Stolz, the bradsot bacillus, and the whale septicemia bacillus. These organisms are extremely varied in their affinities and in their pathologic action; some are proteolytic and some are nonproteolytic. Von Werdt's work is in the nature of a compilation only, and in no way tends to clarify the confusion existing in the anaerobic field. It reflects exactly the history and literature of the subject, and may be consulted for valuable details and for its excellent bibliography. It it not critical.

Foth (b, 252) conceives Rauschbrand to be nonproteolytic and the chain-forming organisms to be proteolytic. His chain-forming strains vary in proteolytic action, but he has never found a chain-forming strain which in its behavior approaches the nonproteolytic character of the bacillus of Ghon and Sachs. Foth uses the term "verbandbildende Anaeroben" to include various species.

Wulff, who worked in Foth's laboratory, has a conception similar to that of Foth and calls all proteolytic infections "malignes Oedem."

Markoff divides his strains, as does von Hibler, into blackleg, nonproteolytic chain formers, and proteolytic chain formers (malignes Oedem).

Weinberg and Séguin (93) declare vibrion septique to be nonproteolytic. Robertson (a) emphasizes the same point. Neither reports any such organisms as the malignant edema of Koch-von Hibler.

Eugen Fraenkel and Zeissler, who carefully isolate their strains, have never found such an organism as the malignant edema bacillus of Koch as described by von Hibler.

In the light of my own experience with anaerobes, I must state that until I find in my hands an indubitably pure culture of a highly proteolytic organism, which alone forms filaments on the liver of a guinea-pig and which has more than a history of pathogenicity, I must continue to doubt the identity, nay, the very existence, of B. oedematis maligni Koch, as described by von Hibler. No one would be more interested or more delighted to find and study such a strain than I. If the organism does exist, I think I am safe in stating that many identifications of it have been faulty, and were based on the investigation of impure cultures. All through the anaerobic literature we find references to pathogenic proteolytic organisms and some of these no doubt exist as entities, capable of penetration in pure culture. Such organisms are, however, exceedingly rare. I have found only two such strains in a series of a hundred tissue invading anaerobes, and these two are only slightly proteolytic. I wish at this point to express my thanks to Miss Muriel Robertson, under whom my anaerobic studies began, for the principles regarding anaerobic investigation which she taught me. It was from her that I learned to discredit the purity of a proteolytic pathogenic culture till I had investigated both the purity and the pathogenicity of such a culture with all the resources at my command. Practically all strains that have come into this laboratory with a history of pathogenesis and that have shown a proteolytic action on meat proved either to be no longer pathogenic for guinea-pigs or to contain a primary invader in the form of a nonproteolytic organism.

I have found no proteolytic invaders in cattle, sheep, or hog material.

I wish also to state at this point that in my hands the characters of anaerobes change no more frequently than do those of other organisms. When a culture radically changes its habit it will be found to contain more than one species of organism.

I wish most categorically to emphasize that: Denaturability of anaerobes in the sense of change of characters as described by Schattenfroh and Grassberger does not exist (a, and Grassberger Arch. f. Hyg. 53). It is only because this preposterous and fanciful conception has been as widely quoted as it has been (Lehmann and Neumann 485; Simmonds, Ghon) that it needs mention. Von Hibler (a, 5) declares that the B. saccharobutyricus immobilis of Schattenfroh and Grassberger is a distinct species from the Rauschbrand bacillus; see also p. 41 of this paper. Zeissler (b) holds Fraenkel's bacillus and that of malignant edema to be immutable species, and insists that mutations of the above kind are due to contaminations. The authors of the Medical Research Committee Report No. 39 (23) recognize the conclusions of Schattenfroh and Grassberger to be erroneous. I believe that other modern investigators of anaerobes will agree with me that the characters of anaerobes are not markedly variable and that work based on the study of mixed cultures is a great hindrance to the progress of science. I have known many cultures to behave as do those of Grassberger and Schattenfroh. Such cultures are always found to be impure.

We may sum up the situation by stating that because of the universal occurrence of organisms of sporogenes affinities and because of their frequent contamination of pathogenic cultures, and because of their occasional invasion of living tissue in company with nonproteolytic invaders, "malignant edema" of animals and man has frequently been described as due to a proteolytic organism.

Friedberger and Fröhner (178) and authors of other textbooks emphasize the universal occurrence of the malignant edema organism, apparently confusing it with the ubiquitous proteolytic forms. They say: (189) "Mit dem Fortschritte der Faulniss verlieren die Oedembacillen ihre Wirksamkeit. Nach etwa 2 Monaten hört sie ganz auf. Trocknet man die Bacillen jedoch bei 15—38 c., ehe Faulniss aufgetreten ist, so bewahren sie ihre Virulenz dauernd." Of the fact that proteolytic organisms so overgrow pathogens as to render them no longer pathogenic or to eliminate them entirely, I could give a hundred examples from my own findings. Weinberg has shown that the filtrate of B. sporogenes neutralizes the toxin of B. oedematiens in vitro and neutralizes to some extent even the toxin of vibrion septique, but that it does not neutralize the toxin of B. perfringens (B. Welchii). Certain workers (Donaldson and Joyce, Mayo-Robson) have even used cultures of organisms of the sporogenes type in the treatment of wounds in man.

Let us for practical purposes group the vibrion septique of Pasteur, the bacillus of Ghon and Sachs, and whatever organisms resemble them. under the name of "the vibrion septique group." A definition of this group is to be found on page 5, b. May I be forgiven if, solely in reviewing the literature, I sometimes use, in conformity with an author, the term "bacillus of malignant oedema" as applying to a proteolytic strain?

Although the traditional idea has been that B. Chauvoei is nonproteolytic and may by this sign be distinguished from B. oedematis maligni, some authors have attributed a proteolytic action to it. Sperry and Rettger refer to the well-known putrefactive organisms B. anthracis symptomatici and B. oedematis maligni. McCrudden states that B. Chauvoei putrefies fibrin. Many older authors do not seem to be very clear on this point, or they make no mention of it. It is a misconception exactly like that concerning the organisms of the vibrion septique group as dealt with by many workers.

(*b*) *Gas Production in the Animal Body*.—This has sometimes been referred to as a distinguishing character. Von Hibler (c. 808) states that Rauschbrand produces more gas in the animal body than does the bacillus of Ghon and Sachs. Lehmann and Neumann (500) state that gas production in guinea-pigs is typical for Rauschbrand and rare for malignant edema. Hutyra and Marek (49) say that more gas is produced in blackleg infections than in malignant edema infections. Wulff (610) states that blackleg cadavers are only moderately enlarged by gas; that gas may occur in the subcutaneous connective tissue but never to a great extent.

Gas production depends on the amount of bacterial proliferation in the tissues, and is a relative matter for each separate wound infection, depending on the site of the wound, its extent and its isolation, and on the toxicity of the specific organism for the specific or individual host and also on the size of the host. The more toxic a strain is for the host, the less bacterial proliferation will there be before the death of the animal, and the less gas will there be formed. Furthermore, a bacterial process may exist longer in a large animal before producing death than it can in a small one. All strains produce gas in meat medium. I find that, given a uniform technic, strains of the vibrion septique group produce distinctly more gas in the muscles and connective tissue of the guinea-pig than is produced by members of the blackleg group. An exception to this statement is the behavior of the vibrion septique group strain which I isolated from whale muscle (p. 85), which is so toxic that it kills before it multiplies to any great extent, and produces little or no gas in the guinea-pig and rabbit. I have observed also that far more gas is to be found in the intestines of guinea-pigs dying of vibrion septique infection than of those dying of blackleg infection.

(*c*) *Formation of Chains or Filaments on the Liver of Animals.*—This has often been used as a criterion of distinction between the two groups. The malignant edema or vibrion septique strains form filaments and the blackleg strains do not. (Friedberger and Fröhner, 180). Von Hibler (a) is most explicit on this point. He also (c, 811) differentiates Rauschbrand from Bradsot, saying that the latter produces filaments and that the former does not. Markoff follows von Hibler in respect to cattle infections. Wulff, although insisting that the morphology of blackleg and malignant edema bacilli shows nothing characteristic in cattle, uses this character as one of his most important ones. Weinberg and Séguin (88) mention vibrion septique strains which do not produce chains on the liver. I have two or three such strains which, however, usually grow out into short filaments if the cadaver is allowed to lie for a day or so. Robertson says (a): "Films made from the peritoneal surfaces of the liver show a greater or less number of long snaky chains and filaments. These long filaments are characteristic of a vibrion septique infection; they are not found in infections with B. Welchii nor with B. oedematiens." As Miss Robertson studied the same strain mentioned by Weinberg as not producing filaments, subsequent to his publication, it is probable that in her hands it did produce filaments. The strain has produced filaments in my hands on the liver of the guinea-pig. I find, however, that the mode of filament production and sporulation after filament production varies with the species of vibrion septique organism, with the dose of culture, with the time elapsing before necropsy, and with the species of animal on whose liver the filaments are produced. I have never observed the formation of chains on the liver of animals dying of a blackleg infection.

(*d*) *Appearance of Infiltration and Necrosis.*—The appearance of the infiltration and necrosis (gaseous hemorrhagic or serohemorrhagic) in the muscles of the host is also used as a differential character. Von Hibler (c, 808) emphasizes the fact that Rauschbrand produces a more markedly hemorrhagic exudate than the Ghon and Sachs bacillus. Hutyra and Marek (49) say: "Besides, the swelling is here more emphysematous, the fluid dark red and foamy, and the muscles are black colored, while in malignant oedema the swelling is more serous, the fluid gray-red or colorless, and the muscles diffusely reddened or only red gray (Gutzeit)." I find the character and degree of the hemorrhagic exudate and the color of the muscle a good lesion to use when standardizing my technic on the guinea-pig. Blackleg almost invariably produces an intense black coloration in spots or in a circumscribed area in the muscles and connective tissue, while vibrion septique strains do not. The latter organisms are, however, distinctly hemolytic in their action, and it would sometimes be very easy for the inexperienced to mistake the coloration produced by them for that called forth by blackleg. It must also be borne in mind that the production of hemorrhagic exudate is a relative matter, dependent on the specific action of the hemotoxin on the corpuscles of the host and on the amount of bacterial proliferation, which in turn is dependent on the location and nature of the wound or focus, on the degree of the toxicity of the specific organism for the specific host, and on the size and age of the host.

(*e*) *Alteration of the Serous Membranes of the Abdominal Cavity.*—This should always be considered in distinguishing between organisms of the vibrion septique group and those of the blackleg group. The tendency to cause injection of the intestines and of the peritoneum is very great in the case of the former and very slight in the case of the latter. This is in the guinea-pig an almost determinative character. Foth (b, 218) and Wulff are emphatic on this point. It has generally been neglected by most workers. I have no data as to what value this character may have in the diagnosis of cattle infections, but the data I have regarding sheep infections make it appear as though it might be a good character for sheep as well as for guinea-pigs (See p. 62). Very highly toxic vibrion septique strains may kill guinea-pigs without producing injection of the alimentary canal (Strain BRH) or even of the peritoneal wall (Strain WS, from whale). The phenomenon is associated in vibrion septique infections with bacterial multiplication.

(*f*) *Presence of the Pathogenic Organisms in the Bile.*—This has been used by a few authors as a means of differentiation. Arloing, Cornevin, and Thomas and Kitt (e) found blackleg organisms abundant in the bile. Von Hibler found blackleg nearly always present in the bile, but he never found edema bacilli or gas phlegmon bacilli there. Scheibel asserts the method is diagnostic for cattle, blackleg entering the bile and edema bacilli not doing so. Foth (p. 212) found this a criterion of no value. Wulff found the method wholly unreliable. He found that in animals whose muscles showed typical blackleg the bile contained organisms which he calls malignant edema organisms, producing malignant edema in the guinea-pig. These infections may have been mixed proteolytic-blackleg infections.

(*g*) *Depilatory Effect of the Inflammatory Process.* — This is a relative character of a little value. It is dependent on the degree of bacterial multiplication in the skin, and is most marked in proteolytic infections, and least marked or quite absent in highly toxic vibrion septique infections. Blackleg infections often show it, and combined with it is frequently a serous exudate. The animals that die most slowly have the greatest bacterial multiplication and their hair is most easily removable.

(*h*) *Delimitation of the Zone of Infection.*—This is not an absolute character. In general, the zone macroscopically affected in blackleg infections is more sharply limited in outline and extent than the zone affected in vibrion septique infections.

(*i*) *Speed With Which Death Ensues.*—This varies of course with the dose inoculated. Given the same technic, almost all vibrion septique strains kill guinea-pigs before the usual time taken for death to intervene in the case of blackleg infections. Guinea-pigs infected with a dose of 0.5 c c of a 24-hour culture of a vibrion septique strain die in about 16 hours, those infected with the same amount of blackleg culture die in approximately 20 hours; but there is great variation in these figures. It must also be borne in mind that a blackleg culture does not contain as many organisms as does a vibrion septique culture.

(*j*) *Odor.* Many authors mention the *characteristic odor* produced in the animal body by blackleg infections. Foth (b, 129) regards this odor as specific. According to Wulff, who has performed many necropsies on cattle dead of blackleg, this odor is to be described as rancidly sour and sometimes also as repulsively sweetish (610), and malignant edema (geburtsrauschbrand) cadavers have a rancidly sour and not a repulsive sweetish odor (690). I should suggest that this character be noted as an aid to diagnosis only when an infection is evidently nonproteolytic, as it is of course of no use for either group if proteolytic or other invaders are present. I have not detected any difference in the odor of guinea-pigs dead of the two diseases. Vibrion septique cultures have the typical rank blackleg odor. Laabs notes the characteristic "blackleg odor" in animals killed by strains which can hardly be accepted as blackleg (see p. 44), while Nicolle, Cesari, and Raphael noted the absence of a butyric odor in guinea-pigs that were killed by their strains, which cannot be accepted as blackleg. Cultures in meat do not seem to differ in odor. I do not think that odor can be regarded as of any real value in differential diagnosis. Jensen (quoted from "Braxy") states that Bradsot cadavers putrefy rapidly. I take this to be the case only in mixed infections. Guinea-pigs dead of vibrion septique infections, on which necropsy examination has been performed and which have been rolled up in paper and left for 48 hours at room temperature show few or no contaminating organisms in smears from the liver and muscles. Even cocci are remarkably rare. This phenomenon is probably due to acid production.

(k) Minor Characters Common to Both Types.—Both types of organisms tend to cause profound liver disturbances in the guinea-pig, which are shown by bile staining of the intestines and frequently by an emptying of the gallbladder. Both types may cause the formation of hemorrhagic erosions on the stomach lining of the guinea-pig, and both may cause enlargement and injection of the suprarenals. Foth (a, 204) and Wulff (45) state that both types produce ochre yellow foci in the liver of cattle. Meyer (c, 684) found infarcts in the liver of cattle dying of atypical B. Chauvoei infection, and (b) isolated vibrion-septique-like organisms from liver foci of cattle dying of an obscure disease in the mountain valleys of California. Both types may invade the pleural cavity and even the lungs; both become septicemic in the guinea-pig, with practically no exceptions, and may be isolated from the heart blood. Both types produce a serogelatinous edema of the connective tissue spaces, and a liquid infiltration of the muscles; they soften somewhat the muscles in which the organisms multiply extensively.

(l) Characters Possessed by Neither Type.—Neither type of organism produces, when inoculated in pure culture, a greenish discoloration of the skin, a foul or putrid odor, tissue disintegration, or a brownish, a grayish, or a yellowish discoloration of the muscles. Neither type produces a gelatinous edema of the connective tissue spaces that retains its gelatinous consistency long after section, nor do they produce an edematous infiltration of the muscles. Though the organisms multiply actively on liver, the phenomenon of "foamy liver" is probably usually a postmortem one.

(m) Pathogenicity for Certain Species.—Pathogenicity for certain laboratory animals, notably for the rabbit, is frequently cited in the older textbooks as a final criterion when one is in doubt as to the nature of an organism under consideration. Malignant edema was thought to be pathogenic for the guinea-pig only in spite of the fact that the bacillus of Ghon and Sachs, generally known to be of the same group as vibrion septique, is not pathogenic for rabbits. Meyer's hog bacillus is relatively nonpathogenic for rabbits, and Meyer (a) emphasizes the fact that "any conclusions drawn from such (pathogenicity) tests would be entirely erroneous without the necessary microscopic examination and anatomic studies." Foth (b, 230) is of the opinion that pathogenicity tests

on various laboratory animals are worthless when performed by the casual diagnostician, but that if they are carefully controlled in the hands of the experienced bacteriologist they are of great value. LeClainche and Vallée, Kitt (e), Wulff (611), and Markoff (221) state that rabbits may sometimes be infected with blackleg. I was, however, somewhat surprised to find that two or more of my indubitable blackleg strains were pathogenic for rabbits, killing them as readily as would strains of the vibrion-septique group, and producing in them the black hemorrhagic muscle lesions. I have also blackleg-group strains that in moderately heavy doses of pure culture sometimes fail to infect guinea-pigs. Markoff finds young guinea-pigs immune to blackleg, but Arloing, Cornevin, and Thomas found them highly susceptible. I have found mice immune to several vibrion septique strains on subcutaneous inoculation of 2 drops of 24-hour meat culture. It must be borne in mind that different mediums and differences in the age of the culture have great influence on the amount of toxin accompanying the organisms. Also in case impure cultures are inoculated vibrion septique strains would be the most ready to infect because they grow better in the presence of other organisms than do blackleg strains. When one considers how greatly the technic of various workers differs in making pathogenicity tests, and when one bears in mind the fact that different individuals of a species vary in their susceptibility to infection, one is ready to agree heartly with Meyer's statement as to the secondary importance of such tests.

(n) Epidemiologic Considerations.—Most deceptive, and, may I venture to say, crudest, of all differentiations between the two groups of organisms are those based on epidemiologic grounds. Diagnosis of the nature of a disease according to whether it is endemic in a locality or whether it is rare in that locality is an unscientific proceeding. Specific diagnosis of an infection, which is based on the presence of a wound as a portal of entry, on the history of a recent parturition, or on the age of the animal attacked, is likewise unjustifiable.

3. CULTURAL AND MORPHOLOGIC DIFFERENCES BETWEEN THE ORGANISMS OF THE TWO PRINCIPAL GROUPS

(a) Motility.—The motility of blackleg and vibrion-septique-like organisms is given differently in different works. Both types (Arloing, Cornevin, Thomas; Pasteur) were originally described as motile. Friedberger and Fröhner speak of B. Chauvoei as nonmotile, and of the bacillus of malignant edema as motile. Meyer's hog bacillus is "actively motile when heated." Weinberg and Séguin (b, 89) state that vibrion septique varies in motility, though they do not state that it is ever highly motile. Robertson states that in 24-hour cultures and in the freshly-taken edema fluid of animals recently dead, vibrion septique is motile. Markoff says that blackleg is slightly but noticeably motile and that the chain-forming type varies in motility. Von Hibler (a, 18) says that both types are motile and that motility varies with circumstances. I find that in an open drop on an unwarmed slide the vegetative rods of the blackleg organism in a 24-hour meat culture may be sluggishly or actively motile, moving in a dancing head-over-heels fashion, while under the same conditions, organisms of the vibrion septique group are noticeably but sluggishly motile; moving occasionally in the manner of a boat or even sidewise, but not tumbling or dancing.

(b) The Gram Stain.—The Gram stain is used to differentiate the two groups. Lehmann and Neumann (500) give malignant edema as gram-negative and blackleg as gram-positive. Bongert says that malignant edema is gram-

negative. Friedberger and Fröhner give both as gram-positive. Von Hibler (a, 13) states that all anaerobes studied by him are gram-positive but that the Ghon-Sachs bacillus and the malignant edema bacillus are the most easily discolored by the alcohol. Foth (a and b) declares both types to be gram-positive. My experience runs counter to most of these statements. I prefer a carbol-methyl-violet (6BX) Gram stain[7] with slight decolorization or a thin carbol-gentian-violet Gram stain, which requires longer decolorization. With either of these stains vibrion-septique strains are usually gram-positive and blackleg-group strains are gram-negative. The Gram stain is of great assistance in anaerobic work. A stain dividing the anaerobic group into gram-positive and gram-negative members is preferable to a thick, heavy, gentian-violet stain that may make the organisms all gram-positive. But care must be taken not to place too much reliance on a complicated stain like this. There are many causes for variation in such a stain. The dye may precipitate; mediums differ in consistency; and organisms may be found to vary in staining reaction on the same smear. One should never call an organism gram-positive unless the surrounding débris of protein has lost the violet stain and the bacillus has kept it. In working with proteolytic anaerobes in meat culture care must be taken to make the film thin, otherwise the débris uses up the violet and there is not enough stain for the bacilli. The Gram stain is of tremendous value in indicating possible contaminations of cultures. The amateur anaerobist should, however, be warned against relying on it as implicitly as he may in the study of aerobes.

(c) The Resistance of Spores to Heat.—This has been used by von Hibler and by some of his predecessors (a, 217) as an anaerobic character. According to him, Rauschbrand spores are more resistant to heat than are the spores of B.VI, B.VII, B. amylobacter, and B.IX. (B. tertius); and they are slightly more sensitive to heat than are the spores of the Ghon-Sachs bacillus, and of B. enteritidis sporogenes Klein; they are distinctly less resistant than the spores of B. phlegmones emphysematoseae, and the Bradsot bacillus (b, 811); and far less resistant than the spores of the Novy bacillus and of the proteolytic organisms. I doubt if spore-resistance could be used to distinguish blackleg strains from vibrion septique strains—and we have many characters to use for differentiation that are more definite.

(d) Morphology.—Opinions vary greatly as to the value of morphologic characters for the differentiation of blackleg from the vibrion septique group. This confusion I attribute solely to the reigning confusion as to the identity of the strains studied. One reason for this state of affairs is that there still are diagnosticians who call an organism blackleg because it comes from a cow. But another important reason is the existence of a large number of contaminated strains in circulation among laboratories. The usual contaminators of sporogenes and bifermentans type, or of tertius or putrificus morphology, naturally upset all morphologic conceptions. Moreover the blackleg organisms in their growth in muscle resemble the vibrion-septique organisms growing in muscle more closely than either type resembles most other anaerobes. This does not mean that they are alike or indistinguishable.

Grassberger and Schattenfroh (c, 889) question all morphologic determinations in this group. This I can readily understand.

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[7] Two and five tenths c c saturated alcoholic solution of stain in 100 c c of 5% pure phenol; dilute 1: 10, filter, apply for 2 minutes, wash, apply Lugol's iodin 1 minute, wash with 96% alcohol, once across the surface of the slide for methyl violet (8 drops), twice across the surface (12 drops) for gentian violet. Counterstain with dilute 1: 10 carbol fuchsin.

Wulff (611), who discusses blackleg excellently from a clinical point of view, insists that microscopic observation is useless, or at most of secondary value, in the diagnosis of blackleg in cattle. Wulff knows blackleg in cattle and I do not, therefore I am not in a position to contradict him. He bases his statement on the fact that blackleg is highly polymorphic and on the fact that the contaminating organisms in the cadaver are confusing. He finds the microscopic picture in the guinea-pig of more use, however, and employs the formation of chains on the liver of that animal, as well as on the liver of cattle, as one of his most important criteria of differentiation.

Von Hibler knew Rauschbrand, and he knew the vibrion-septique group under the name of the Ghon-Sachs bacillus. In "Untersuchungen über die pathogenen Anaeroben" (26, and in various photomicrographs) and in "Rauschbrand" (792) he emphasizes the morphologic distinctions between the groups.

Miessner and Lange find morphology of considerable value in the diagnosis of blackleg.

I may sum up the value of morphologic determination according to my own experience as follows: If the worker is unfamiliar with anaerobic technic and with the anaerobic group as a whole, his opinion (beyond his observation of filaments on the liver of an animal) is worthless. And if the worker has not an eye capable of distinguishing with some certainty a contamination in a smear of an anaerobic culture in vitro or in an animal, his opinion is worthless. But for the trained anaerobist, employing a technic with which he is familiar, the organisms of these two groups are, in morphology and in staining reaction, highly characteristic, and they are not to be confused. I could undertake to teach any observing bacteriologist the difference between the two types in a few hours. I would suggest that those interested in this subject study carefully pure cultures of organisms of the two groups in the guinea-pig and in meat medium with a weak or carefully decolorized Gram stain (see p. 19, b). Forms are occasionally seen which may be referred to either group; morphologic criteria could not free a suspected mixed blackleg-vibrion-septique culture of suspicion, but the general picture of the cultures and of the smears of animal exudates containing these two types is entirely different, and any one can recognize this difference on his own mediums and with his own stain as soon as he is acquainted with both types.

(e) *General Cultural Characteristics* are not of great value in distinguishing the two groups. In general the organisms of the blackleg group are not so active in their growth on artificial mediums as are those of the vibrion-septique group; they occasionally fail to grow on a medium on which vibrion-septique organisms will readily grow. For example, I have repeatedly failed with massive inoculation to induce them to grow on brom-cresol milk. But in gross biologic determinations the two groups are so closely related that they are thrown together rather than separated. However, gross determinations separate these groups from other groups, especially from the Welch group and from the oedematiens group. Finer biologic determinations may be of great value, not only in differentiating the blackleg organisms from the vibrion-septique organisms, but also in differentiating distinct species within these groups. So far as my experience in the matter goes, the more detailed and more or less quantitative cultural tests, combined with morphologic characters, are of more value in differentiating these organisms than are immunologic tests.

(f) *Colony Formation.*—Colony formation has not been used by many recent authors as a means of differentiation between the two groups. Regn found both rapidly-growing spherical colonies and slow-growing lenticular colonies to be characteristic of what he termed Rauschbrand.

Kitt (e, 291) describes malignant-edema colonies in agar as follows: "Die Kolonien sind hier unregelmässig, bald nur äusserst zarte punktförmige, graue Fleckchen, bald dickere, drusige, stachelige hirsekorngrosse Herde (vielleicht nach Stämmen odor nach Zusammensetzung des Agars varrierend)" and blackleg colonies are spoken of as follows (e, 300): "In Agar erblickt man schon nach 12-36 Stunden bei Züchtung im Brutofen die entstehenden Kolonien als zarte, drusige Punkte und eine starke Gasbildung, durch welche der Nahrboden in Stücke zerspellt, hochgeschoben und allenfals der Watteprof herausgetrieben wird," and he depicts them as fluffy and spherical. I do not claim that in some mediums blackleg organisms may not form spherical, fluffy colonies. I have, however, found colony formation in agar under carefully controlled conditions to be of immense value in distinguishing, not only between the blackleg and vibrion-septique groups, but also between the individual species in these groups.

Von Hibler, whose book was in the hands of Regn, clearly defines the Rauschbrand bacillus (a, 57, ff.) as forming in deep agar lenticular or spherical smooth colonies, which only locally and occasionally show smooth or irregular protuberances on the surface. His illustration (a, pl. II, fig. 5) shows colonies exactly typical of those formed by most of the blackleg strains in my hands. He quotes Kitasato (55) as describing the colonies of Rauschbrand as follows: "'unregelmässige Kugeln mit leicht warziger Oberfläche' also doch immerhin als geschlossene Gebilde," and he represents Sanfelice (pl. IV, figs. 38 and 40) as picturing Rauschbrand colonies as "durchweg Linsenformen, die aber zum Teil unter Aussendung von wurzelartigen Ausläufern odor infolge örtlichen Hervorbrechens von Vegetationsmassen ihre Geschlossenheit und glatte Begrenzung verloren haben." Von Hibler states that the bacillus of Ghon and Sachs belongs to a class that forms colonies that early show irregular clumps with rootlike or tufted projections, that is, they are "zerschlissen," and if they are at first smooth, they later form mosslike projections all over their surfaces. His cuts (a, pl. II, figs. 12 and 13) show them to be large fluffy colonies. He quotes Ghon and Sachs as describing their organism as forming such colonies. In his monograph on Rauschbrand (c, 798) he is positive in his statements as to the usual lenticular form of Rauschbrand colonies.

Foth (b, 134) emphasizes the value of colony formation as a differential character (see p. 46).

Meyer and Haslam (Meyer, c) both mention the colony formation of blackleg as a valuable character.

(g) *Carbohydrate Fermentations.*—Carbohydrate fermentations have occasionally been resorted to as a means of differentiating anaerobes.

Achalme analyzed the behavior of vibrion septique and the bacillus of symptomatic anthrax on sugars. As his cultures were proteolytic I shall not quote his results.

Smith used fermentation tests on three sugars to distinguish his anaerobic strains. The nonproteolytic strains called "Rauschbrand" behaved consistently; the proteolytic strains called "edema bacilli" behaved differently from the nonproteolytic ones and consistently with each other.

Bahr made a more extensive study. He divided his strains into 5 groups, the first group containing 9 strains of quite different origin: sheep, hogs, cattle and a horse; they were from different countries. The other groups contained one strain each. Bahr isolated his strains by animal passage and a colony method.

Grosso studied carbohydrate fermentations. His isolations were carefully made.

Meyer (a) found his hog strain, a vibrion-septique strain, and a blackleg strain to behave alike.

Todd worked out an elaborate scheme for sugar determinations on this group, but some of his cultures were proteolytic and the work cannot be quoted.

Robertson found vibrion-septique strains behaved alike, and a B. Chauvoei strain behaved differently from them.

Christiansen made extensive determinations on his whale septicemia bacillus.

One observes a general tendency on the part of anaerobes of these groups to split the hexoses and the disaccharids, and to leave the higher carbohydrates untouched. Smith's nonproteolytic group, Bahr's group 1 (9 strains), Grosso's group 1, Meyer's 3 organisms, Robertson's vibrion septiques, and the whale septicemia bacillus of Christiansen are wholly consistent, and may be taken as characteristic of the vibrion-septique group. They may be signalized as not splitting saccharose. The remaining strains referred to B. Chauvoei are not so consistent in their behavior; Grosso's and Robertson's strains split saccharose. There are not enough data from which to draw further conclusions. I hope to perform extensive fermentation tests with my strains in the near future. It would appear that a satisfactory basic medium for carbohydrate determinations for use with anaerobes is yet to be found.

4. DIFFERENTIATION BETWEEN THE TWO PRINCIPAL GROUPS BY MEANS OF IMMUNE REACTIONS

(*a*) *Immunization in General.*—The anaerobes with which we are dealing are antigenic organisms par excellence. Immunization experiments have been carried out by many workers, in almost as many ways. Results are apparently contradictory, but on careful scrutiny they are found to be consistent. Immunization of guinea-pigs has been accomplished with cultures, washed or heated organisms, and organisms chemically attenuated; with toxic and even nontoxic filtrates of cultures and of infected tissue; also with serum from immunized animals.

(*b*) *Active Immunization with Cultures.*—We shall deal here chiefly with immunization work which was performed with the purpose of distinguishing our two important types. I find the following notes as to the active immunization of laboratory animals by means of cultures:

Roux in 1888 used culture which had been heated at 115 degrees for immunization purposes.

Kitasato in 1889 found that a two-weeks-old culture of B. Chauvoei in guinea-pig broth could be used for the immunization of guinea-pigs, as could a heated culture.

Le Clainche and Vallée found that guinea-pigs immunized by attenuated blackleg virus were not immune to vibrion-septique infection.

Markoff used cultures in 0.5% sodium formate broth with 2-5% glucose and found immunization of guinea-pigs with blackleg and chain-forming organisms possible with such cultures.

Wulff (612) used dry powdered infected muscle for immunization.

TABLE 1

Table of Carbohydrate Fermentations as Quoted from Various Workers *

	Glucose	Levulose	Galactose	Saccharose	Maltose	Lactose	Raffinose	Mannose	Xylose	Rhamnose	Arabinose	Amylum sol.	Inulin	Cellulose	Glycogen	Salicin	Amygdalin	Trehalose	Mannite	Dulcite	Quersite	Sorbite	Glycerin	Adonite
Smith's nonproteolytic	+	..	..	—	..	+	..	..	..	..	..	..	..	..	..	..	..	..	..	..	.	..	..	..
Smith's proteolytic	+	..	..	—	..	—	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
Bahr's group I	+	..	..	—	+	+	..	..	—	—	—	..	..	..	..	..	..	..	—	—	..	—	—	—
3 Bradsot																								
2 Oedematis maligni																								
1 Pseudo-Rauschbrand horse																								
3 Rauschbraod cattle																								
Bahr's group II	—	..	..	—	+	+	..	..	—	—	—	..	..	..	..	..	..	..	—	—	..	—	+	—
Rauschbrand, Kitt																								
Bahr's group III	+	..	..	+	+	+	..	..	—	—	—	..	..	..	..	.	..	..	—	—	..	—	—	—
Bradsot, Scotland																								
Bahr's group IV	+	..	..	+	+	+	..	..	+	—	+	..	..	..	..	..	.	..	+	—	..	+	+	—
Rauschbrand, Ostertag																								
Bahr's group V	+	..	..	+	+	+	..	..	+	+	+	.	..	..	..	..	..	..	+	+	..	+	+	—
Malignant edema, Halle																								
Grosso's group I	+	..	..	—	..	+	..	..	—	—	—	..	..	..	..	..	..	..	—	—	..	..	—	—
2 Malignant edema																								
1 Bradsot																								
Grosso's group II	+	..	..	+	..	+	..	..	—	—	—	..	..	..	..	..	..	..	—	—			—	—
2 Pseudo-Rauschbrand																								
Grosso's group III	+	..	..	+	..	+	..	..	—	—	—	.	..	..	..	..	..	..	—	—	..	..	+	—
Rauschbrand																								
Meyer's hog bacillus	+	..	..	—	+	+	..	..	—	—	—	..	..	.	..	.	..	..	..	—	..	—	—	
Vibrion septique																								
Rauschbraod 2, Munich																								
Robertson, several Vibrion septiques	+	+	+	—	+	+	..	..	..	..	..	..	—	..	..	+	..	..	—	—	..	.	—†	
Robertson's B. chauvoei	+	+	+	+	+	+	..	..	..	..	..	..	—	..	..	—	..	..	—	—	.	..	—†	
Christiansen's whale septicemia bacillus	+	+	+	—	+	+	—	+	—	—	—	..	—	+	—	+	—	+	—	—	—	—	—	—

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* Corrected copy not identical with Journal copy.
† Med. Res. Committee, No. 39.

The authors referred to above all agree that blackleg and the chain-forming organisms may be distinguished by this means.

(*c*) *Types of Toxin.*—The best known anaerobic toxins are those of the proteolytic organisms B. tetani and B. botulinus. These toxins are apparently produced slowly in a medium, and increase in power as incubation continues. They are pathogenic in extremely minute doses. It is of interest that the more proteolytic a pathogen is, the more powerful is its toxin, the less energetic its invasive properties. The toxin of B. oedematiens, which has mild proteolytic properties (Med. Res. Comm. No. 39, 63), is less powerful than that of B. tetani and that of B. botulinus which is the most highly proteolytic of the three, but it is far more powerful than that of the vibrion-septique and Welch-group types, which are nonproteolytic organisms (Med. Res. Comm. 39, 116).

(*d*) *The Aggressin Test.*—The determination of toxin and antitoxin relationships is of immense practical value. Tulloch has shown in his tetanus work that aggressins are antigens which must also be considered in anaerobic infection. Probably cultural methods can be made to supersede the agglutination method entirely, because immunologically it has no practical significance. But the toxins and the aggressins have great practical significance: What does it matter to the therapeutist if two strains can be shown to belong to two different species by colony formation or other delicate methods, so long as they produce the same toxin and aggressin? A practical method of arriving at the combined toxin-aggressin factors would be by means of the immunization of guinea-pigs with young whole culture, after a preliminary inoculation of killed organisms. More delicate methods of demonstrating aggressins are two: (1) The delicate phagocytic methods in use in Sir Almroth Wright's laboratory may be used to show the action of bacterial products and their antibodies on leukocytes; or (2) accurate animal experiments may be turned to, filtrates of definite age from mediums of standard composition being used.

Immunization by means of filtrates was undertaken in 1888 by Roux, who found that filtrates of cultures of vibrion-septique and B. Chauvoei were excellent for immunizing guinea-pigs. He found filtered serous exudate of guinea-pigs dead of vibrion-septique infection to be more highly toxic for guinea-pigs than that of guinea-pigs dead of blackleg infection, and he found such serosity to be a good immunizing agent. By such a means he immunized guinea-pigs against both diseases and found that those immunized against vibrion septique were not immune to symptomatic anthrax, but that those immune to symptomatic anthrax were immune to vibrion septique. Kitasato repeated Roux's experiment and found that Rauschbrand did not protect against malignant edema.

Schöbl (1910) found that centrifuged edema fluid of an animal dead of blackleg infection immunizes guinea-pigs against blackleg inoculation. This method has received wide practical application in the immunization of cattle.

Nitta (1918) found that blackleg culture filtrate furnished adequate protection to cattle.

(*e*) *Passive Immunization.*—More extensive work has been done in the way of differentiation by means of passive immunization.

Duenschmann, in 1894, attempted to demonstrate immunologically the relationship between vibrion septique and B. Chauvoei. His immunization strain, called B. Chauvoei, which he received from Arloing, who used it for vaccine, was, however, a member of the vibrion-septique group. This was pointed out by Le Clainche and Vallée (595). Duenschmann considered it to be a true

B. Chauvoei because it did not kill rabbits. It killed guinea-pigs rapidly "with all the well-known signs of symptomatic anthrax" (details not given); it sporulated heavily in the animal body, many spores being median; the bacilli were short, but formed long motile filaments on the peritoneal wall. Duenschmann found that the serum of rabbits immunized with blood cultures of this strain immunized guinea-pigs against a stock vibrion-septique strain of the Pasteur Institute.

In 1900 Le Clainche and Vallée studied immunologically the relationships between B. Chauvoei and vibrion septique. They found that a B. Chauvoei serum which protected guinea-pigs against B. Chauvoei inoculation, would not protect against several strains of vibrion septique, all of which were protected against by one vibrion-septique serum.

Foth (a, 254) used serum of rabbits immunized by injection of cultures of Rauschbrand bacilli. This was given subcutaneously to guinea-pigs in one thigh, culture was given on the other; and animals that were protected, while controls died with lesions of Rauschbrand, were considered to prove the existence of Rauschbrand.

Kitt (a) says that serum immunization may be used to differentiate Rauschbrand from the infections that simulate it.

Markoff preferred serum immunization of guinea-pigs to culture immunization, and found that it divided his organisms into fundamental groups.

Wulff (612) found serum immunization a diagnostic aid of great value.

Differentiation of blackleg from malignant edema by means of toxin-antitoxin determination has been undertaken by Grassberger and Schattenfroh (b and c). They hold this procedure to be the only fundamental means by which to distinguish the two types from one another, which is entirely comprehensible when one considers their technic. They use an inoculum of dried juice from an animal dead of blackleg, which is confessedly impure, which Grassberger and Schattenfroh hold to be unimportant. They then place a piece of this material in a tube containing a chunk of "sterile" beef muscle, taken aseptically from a larger piece (Foth [b, 241] and Conradi claim that such muscle is seldom sterile), pour over it 2-3% glucose broth, incubate in a Buchner tube and in 24 hours find that microscopic examination shows sporulating and nonsporulating forms of "half denatured" Rauschbrand bacilli! They find that this broth is highly pathogenic (unfiltered?) for rabbits, and that a highly potent antitoxin will protect the rabbits. Likewise, malignant edema forms a toxin which is specific.

I have every confidence that the toxins of blackleg and of vibrion-septique strains will be found to be specific—what we need to know is whether all toxins produced by the members of one group are alike (see p. 57). I wish, however, most emphatically to protest against such gross methods as those of Grassberger and Schattenfroh. Immunologic work cannot be regarded as serious when it is performed with impure cultures. It is especially necessary to have pure cultures with which to produce immune serums, but Grassberger and Schattenfroh do not mention any precautions taken in this matter. Especially is this fact to be considered as entirely disqualifying the work of Grassberger and Schattenfroh when one considers the fact that the usual "denatured form" of Rauschbrand described by Grassberger and Schattenfroh is the ubiquitous B. Welchii (B. phlegmones emphysematoseae) [von Hibler a, 5], and that that organism produces a highly potent toxin in the type of medium described by Grassberger and Schattenfroh.

Meyer (a) found that serum immunization sharply distinguishes the groups.

Nicolle, Cesari, and Raphael demonstrated by means of antiserum the identity of the toxins of various tissue-invading strains of animal origin. They found also that their antiserums protected against cultures of the various strains. These strains comprised one strain from the blood of a cow, which was putrified, one from the cadaver of a rabbit "altéré," and two from the cadavers of guinea-pigs "altérés"—these strains Nicolle, Cesari, and Raphael called vibrions septiques. The authors studied also several strains that they call B. Chauvoei, one of which came from a hog and the others from cattle. I can find, however, no evidence in their protocols that they worked with any typical strains of B. Chauvoei. They repeatedly insist that their strains all behave alike except for some differences in pathogenicity, the organisms all kill rabbits if the dose given is as much as 1 c c, and all apparently produce "bulles gazeuse" in the animal body, and congestion of the intestines; the black hemorrhagic exudate so typical of blackleg is not mentioned. It is impossible to believe that differences so striking as those noticed in the pathology of the two diseases in the guinea-pig body would have been passed over by these workers had they been seen. The identification of the strains of hog and cattle origin was probably referred to the donors of the cultures. Three of these strains were from Dr. Vallée. I find that one of Vallée's classic B. Chauvoei strains belongs to the vibrion septique group. I am at a loss to account for this as it is apparent that Le Clainche and Vallée were entirely aware of some of the distinctions between the two types. Le Clainche and Vallée state, however, that it is difficult to keep a strain of B. Chauvoei from becoming displaced by vibrion septique through animal passage. I have never known this to happen with my technic, though it is quite conceivable that it might happen if peritoneal or subcutaneous serous exudate were used for purposes of inoculation.

A recent piece of work is that of Klose (c). Klose studied the protection afforded to mice and guinea-pigs against the toxins of anaerobes by antitoxic serums. The purity of Klose's strains would bear investigation. He divided them into two groups, the putrificus (proteolytic) group, and the Rauschbrand group; in the latter are included all strains which formed a toxin that was neutralized by a "Rauschbrand" serum of the "Höchster Farbwerke," and also the organisms which culturally resemble the Ghon-Sachs bacillus. His results show that the Höchst "Rauschbrand" serum must have been made from a vibrion-septique strain. Klose investigated 4 strains and 5 serums. Two strains were distinctly proteolytic (K16b and KI); one (Ficker I) partially liquefied serum but was relegated to the nonproteolytic group, and one was nonproteolytic (Berlin). The serums "Gas-Oedem Höchst Op. 3," "Rauschbrand Höchst," and "Goldfuchs" from v. Wassermann, all protected against the nonproteolytic strain, the slightly proteolytic strain, and one of the highly proteolytic strains. They did not protect against the highly proteolytic strain KI, and the antitoxin of strain KI did not protect against the other strains. The pathogen in strain KI may be assigned to the oedematiens group, as it resembles B. Novyi (a). Landau showed that the Höchst serum did not agglutinate two of Kitt's Rauschbrand strains. I doubt if any true Rauschbrand entered this complex. The matter of proteolysis I lay to contaminations. Toxin determinations will probably prove to be excellent for the practical differentiation of these organisms.

Klose (a) studied elaborately the toxin of a highly pathogenic anaerobe that resembles the B. oedematis maligni II of Novy. He insists (b, 293) that immunologic methods, especially toxin-antitoxin determinations, are fundamental criteria for specific determination of anaerobes. Zeissler (a, 489) finds Klose's strains of pathogenic anaerobes contaminated with proteolytic organisms and attacks Klose for placing "putrificus" strains in the Rauschbrand group on the basis of toxin-antitoxin determinations. Zeissler calls the value of toxin-antitoxin determination seriously into question. Of course, if one is to use impure cultures in such determinations one should not make the mistake made by Klose of confounding the cultural characters of the contaminators with those of the toxin-forming organisms.

Various workers have produced toxins from the organisms of the groups under discussion, though Haslam and Lumb showed that blackleg culture filtrates made by four different laboratories possessed immunizing properties but no toxic power for the guinea-pig. These authors were careful as to the identity and purity of their cultures. I should not consider that their work shows that blackleg organisms do not produce toxin, since the cultures used were not young ones.

(*f*) *The Agglutination Test.*—The agglutination reaction has naturally frequently been utilized as a means of differentiating anaerobes.

Le Clainche and Vallée sharply differentiated B. Chauvoei from vibrion septique by means of the agglutination reaction.

Hillbrand differentiated Bradsot from malignant edema by agglutination.

Grosso discusses the question. He found that all bradsot strains were agglutinated by the same monovalent serum, which also agglutinated all his malignant-edema strains. He therefore pronounced the malignant-edema organisms to be the cause of Bradsot. His agglutinations were consistent, as follows:

Bradsot serum agglutinated malignant-edema cultures.
Malignant-edema serum agglutinated bradsot cultures.
Rauschbrand serum did not agglutinate bradsot cultures.
Rauschbrand serum did not agglutinate malignant-edema cultures.
Malignant-edema serum did not agglutinate Rauschbrand cultures.
Malignant-edema serum did not agglutinate pseudo-Rauschbrand cultures.
Rauschbrand serum agglutinated Rauschbrand cultures.

Grosso defines as pseudo-Rauschbrand a type of organism that resembles Rauschbrand closely and is pathogenic for mice; it does not form chains. Grosso isolated his organisms from the heart blood of guinea-pigs.

Markoff used the agglutination reaction to distinguish his strains which were of cattle origin. The filament-forming strains fell into one group and the nonfilament-forming ones into another.

Wulff (612) found the agglutination of cultures to be of value in distinguishing Rauschbrand from diseases that resembled it. The serum of cattle dead of Rauschbrand contains, however, no agglutinin.

Meyer (a, 458), made use of the agglutination reaction for the identification of his hog bacillus. The serum made with the hog bacillus agglutinated to a high titer a vibrion-septique strain of the Pasteur Institute and to only a low titer the blackleg and malignant-edema strains which were available.

McIntosh found that the agglutination reaction distinguished sharply between the groups of anaerobes. A B. Chauvoei serum agglutinated its own strain to a high titer and a vibrion septique strain to a low titer, and the serum of the vibrion-septique strain agglutinated the homologous strain to a high titer and the B. Chauvoei strain to a low titer.

The Anaerobe Committee recommend agglutination as a means of distinguishing the groups of organisms.

Robertson (b) found that the vibrion septique strains studied by her formed four groups on the basis of the agglutination reaction and she found that impure or recently isolated strains frequently failed to agglutinate. She considers a positive agglutination with a vibrion septique serum to be specific.

Zeissler (a, 110) asserts that he has found Rauschbrand 10 times as a human wound infection. Agglutination with a serum derived from a "Rauschbrand" (?) strain from a cow was the determinative measure used. (In 6 of these cases Fraenkel's bacillus was also present.) Zeissler does not say whether the wound infections were gangrenous processes, or whether he isolated the organism merely from septic wounds. He says (b, 40) that the Ghon-Sachs bacillus so closely resembles the Rauschbrand bacillus that there is no fundamental or morphologic difference between the two, except that the former makes filaments in the animal body. As Zeissler worked with a colony method that should have distinguished true Rauschbrand from Ghon-Sachs-like organisms, I am forced to suspect that his "Rauschbrand" used for the manufacture of the agglutinating serum was an organism of the vibrion-septique group that showed little tendency to form filaments in the animal body, or perhaps that Zeissler did not look at smears from the peritoneal cavity to find filaments. This suspicion mounts almost to certainty because Zeissler found the bacillus of Ghon and Sachs only once in man and "Rauschbrand" 10 times.

Zeissler (a, 110) states that two anaerobe strains, which were manifestly of a different species from that with which a serum was made, were agglutinated by that serum. He guarded against auto-agglutination. He heartily condemns the agglutination reaction as a means of differentiation of anaerobes and calls into question all decisions that rest on it.

Passini finds that putrificus-immune serums may agglutinate organisms of another species to a higher titer than that to which they agglutinate other strains of putrificus. He intimates that this finding strengthens the thesis of Grassberger and Schattenfroh, namely, that the anaerobes are all more or less the same. I should suggest, instead, that the data of Passini show the worthlessness of the agglutination reaction, at least as practiced by him.

Robertson (private communication) immunized a rabbit with a strain of my own (B. II, Hempl) that is to be assigned to the bifermentans group. The serum of this rabbit agglutinated B. sporogenes. I do not think there is any reason to doubt the purity of the strain used by Miss Robertson.

My experience with agglutination in the differentiation of anaerobes has been distinctly unsatisfactory. I heartily agree with Zeissler in his condemnation of it. The phenomenon of auto-agglutination interferes frequently: in the oedematiens group it makes all agglutination work impossible; in the blackleg group it often gives trouble but may be eliminated by adjusting the electrolyte content of suspensions and by adjusting the P_H reaction; in the vibrion-septique group it causes little inconvenience. But there is a variable element in the reaction, existent in the cultures of the organisms to be agglutinated, which lies always ready to trap the unwary. I find that vibrion-septique strains fall into several groups on agglutination, as stated by Robertson (b); but when the experiment is repeated most of the strains will behave as they did before; one or two of them may behave, however, in so different a fashion that the worker is at first led to suspect that he has made mistakes in his technic, and is later forced to conclude that the medium and technic used are unsatisfactory for the problem in hand. The medium used by myself was a casein-digest broth containing a chunk of liver. All digest mediums have now been discarded and others are being tried. It is to be hoped that a technic and medium may be developed that will furnish regularly reproducible results. With a given serum some strains agglutinate to a high titer and some to a low titer. The phenomenon of an inhibition zone was also observed. Several strains are agglutinated by one serum while another serum agglutinates only one of them. One strain was agglutinated by serums of the three principal types. The agglutination reaction may in general be used to separate one group from another, but we have plenty of other criteria for such separation that are easier to use and more dependable. The Medical Research Committee (39, 55) term the agglutination reaction "ultra specific." For the therapeutist this statement is probably correct, but for the systematist and for the epidemiologist the finer differentiations are of great value. It is to be hoped, therefore, that absorption-of-agglutinin tests may lead to some advance in breaking up the groups of anaerobes. Agglutination tests in the anaerobic group are not to be undertaken lightly with a few strains, and I do not believe that agglutination tests performed with impure cultures can be of value, at least not in the vibrion-septique and blackleg groups. It should be remembered that the use of another much-heralded immune reaction, namely phagocytosis, has been largely abandoned because of occasional inexplicable inconsistencies in its behavior.

(*g*) *The Precipitin Test.*—In large veterinary institutes in countries like Germany, where losses by Rauschbrand are recompensed by the government, the problem of determining the nature of such a disease cheaply and expeditiously from pieces of flesh sent to the laboratory is a serious one. Miessner and Lange propose the precipitin reaction as a solution for such a problem. They successfully immunized horses to Rauschbrand and to malignant edema. The precipitin reaction was carried out on the juices of the muscle of the animal in question. It failed or gave an equivocal result in 14 out of 65 cases that were anatomically and microscopically referable to Rauschbrand. But Miessner and Lange do not tell us what results these cases gave with a malignant-edema precipitin, nor do they give us other details as to the strains of organisms found in these cases. I think it very likely that the material that showed no precipitation with a Rauschbrand serum may have been from animals with an infection by an organism of another group, and thus worth investigating. One point must be suggested: Perhaps precipitin reactions on extracts from pieces of Rauschbrand muscle will work best for low virulent infections and less markedly for highly toxic infections because such reactions are probably dependent on the number of bacilli in the muscle. Miessner and Lange note great variation in the intensity of the reaction.

Ascoli and Valenti found the precipitin reaction of great use in the diagnosis of anthrax. Pfeiler says that anthrax is the disease par excellence whose diagnosis may be established by this reaction. He quotes several authorities who used it for medicolegal determinations on badly decayed cadavers that were entirely unsuited to microscopic or bacteriologic examination. He and Rehse obtained positive results with a sample of mud from a spot where a cow, sick of anthrax, had been slaughtered weeks before.

(*h*) *Hemolysins.*—Nicolle, Cesari, and Raphael showed that the hemolysins in 5-day Martin's broth cultures of their strains showed variations which were of no differential value. I find all organisms of the tissue-invading groups highly hemolytic in their action in blood broth.

(*i*) *Hemagglutinins.*—The same authors found that all these strains gave hemagglutinins for guinea-pig red blood cells, and that four of their B. Chauvoei strains and one vibrion-septique strain gave hemagglutinins for rabbit red blood cells.

(*j*) *Leukocidins.*—Eisenberg found that a Rauschbrand antileukocidic serum was as effective against the leukocidin of B. oedematis maligni as against that of B. anthracis symptomatici. Eisenberg does not present details as to the identity of his strains.

(*k*) *The Complement-Fixation Test.*—Rocchi found complement fixation to distinguish anaerobes of various strains. Even different strains of the same name were not identified by this reaction. The collection was one of strains from various laboratories, and details as to their behavior are not given.

Ascoli and Valenti could find no application for the complement-fixation reaction in anthrax material.

Summary of Uses of Immunologic Tests.—It may be concluded that for separation of the organisms of the blackleg group from those of the vibrion-septique group various immunologic methods may be employed. I should place these in order of preference with regard to convenience and value as follows: Immunization of guinea-pigs by serum, by toxin, by cultures; precipitation; agglutination. I should hesitate to classify the other methods.

Plenty of suggestions may be given the beginner, however. First and foremost he must beware lest he be dealing with a mixed infection. Then in all probability the vibrion-septique group may be broken up into subgroups by some other immunization procedures, as it may by the agglutination reaction, and the blackleg group may probably also thus be broken up; superficial work might thus lead to an absolutely false conclusion. Two test animals and two controls should be used for each experiment. Young guinea-pigs are said to be immune to blackleg.

I venture to say that immune reactions will in time automatically be relegated to the province of the serum laboratory where the antigenic status of the organism is its only interesting feature. We have or can develop abundant other methods for classifying the anaerobes according to their group affinities, and we have cultural methods that divide up the groups into smaller and more readily recognized subdivisions than do immune reactions.

II. An Investigation of Material Derived from Animals

By isolating organisms from animal material and from cultures derived from animals and by securing strains already isolated by others I have made a collection of 54 strains of pathogenic tissue-invading anaerobes. A classification of these strains according to their group affinities and according to their source is excerpted from the appendix.

1. PATHOGENIC STRAINS DERIVED FROM ANIMALS

Strains of the Vibrion-Septique Group 30: from cattle 13, from sheep 8, from horses 2 (one spontaneous infection, one accidental laboratory infection with a human strain), from hogs 2, from guinea-pigs 2, probably from guinea-pigs or rabbits 2, from whale 1.

Strains of the Blackleg Group 21: from cattle 19, typical, 15, atypical (probably none identical) 4, from sheep 2, one apparently typical and one atypical and unique.

Organisms of the Oedematiens Group, 3: from horses. Three edema producing organisms of the same species referable to the oedematiens group were specifically distinct from all other strains in my collection.

From a few specimens of original material from cattle and sheep I was unable to isolate pathogenic organisms—the material was probably too old. I much regretted this in the case of the sheep material from Professor Hamilton which was given me by Dr. Jensen, because Hamilton's material is in a way classic and has been studied by several workers. From a sadly large proportion of cultures it was found to be quite impossible to isolate a pathogenic organism. Proteolytic contaminations had completely outgrown the original pathogen. Still, a fairly large number of pathogenic organisms was recovered.

2. TECHNIC OF ISOLATION

The technic employed in isolation was, briefly, as follows:

Muscle material or culture received was inoculated into tubes of meat medium and was incubated anaerobically for about 20 hours. It was then examined. If the flora comprised aerobes, the culture was heated in a pipet and inoculated into fresh medium. If, on examination, the flora was apparently highly proteolytic, a large dose of 1.5-2 c c was injected into the thigh muscle of a guinea-pig; if the flora was nonproteolytic and suggested the presence of a known pathogen, a small dose of about 0.5 c c was injected into the thigh muscles of a guinea-pig. To avoid contamination of syringes by sporulating organisms, Pasteur pipets have been used for inoculations. This is the method used by von Hibler. Cultures were recovered from the heart blood after the death of the animal and were studied critically to detect contaminations. Further isolation was accomplished early in the period of the investigation by Barber's single bacillus technic (usually more than one bacillus from an apparently pure culture being used), and later by my usual deep colony method. All cultures were checked several times for purity by a deep colony method. When a culture with a history of pathogenicity was found to be nonpathogenic for a guinea-pig, it was inoculated (20-hour culture) into another guinea-pig with a small drop of lactic acid. If it then failed to infect, it was inoculated onto liver broth and the same technic was employed as for meat medium. To this type of perseverance I owe the unique and highly pathogenic strain AS. If no pathogenicity was shown after these efforts, the material was abandoned.

An interesting feature was that in three cases in which a mixed infection of a vibrion-septique type of organism and of a blackleg type of organism existed in the material from the original cow, as could be easily detected by the morphology of the organisms in the meat medium, a culture of the mixture on meat medium gave a vibrion-septique infection in the guinea-pig, and a culture on liver broth gave a blackleg infection in the guinea-pig. This could probably not be accomplished with all such mixtures, as vibrion-septique strains are known to differ considerably in pathogenicity. I know, however, of no other way, short of immunization of guinea-pigs, whereby blackleg may be isolated when a vibrion-septique strain is present.

This is an expeditious method for isolating pathogens. I do not pretend that I isolated all the pathogenic organisms in the material at hand. It is, however, probable that all the vibrion-septique strains present were isolated. I know of no cultural method of isolating blackleg from a gross mixture. Its colonies are so slow to develop that it would seem almost a hopeless task to try a colony method to isolate it unless it were in predominance. Vibrion-septique organisms may, however, easily be isolated from mixtures by a colony method.

3. DISCUSSION OF TECHNIC OF ISOLATION AND OF SOURCE OF MATERIAL

I am well aware of what some of my critics will say concerning this method of isolation. Foth emphasizes repeatedly that carefully dried muscle of animals dying from blackleg should be the material used in inoculating the guinea-pigs by whose aid the primary isolation is made. Otherwise, says he, contaminating organisms may also infect the guinea-pig. Contaminations are of three kinds: nonsporulating organisms, which I always kill by heat before the culture used for inoculation is grown; sporulating organisms capable of penetrating tissue in the company of another organism only; and sporulating organisms of high pathogenicity. The two latter groups Foth lumps in general together as "verbandbildende Bakterien." If one has only one guinea-pig to use per strain it would be unwise to give it a heavy dose of mixed anaerobic culture because proteolytic forms might reach the heart blood and the culture therefrom be impure. But may I assure the reader that the element of proteolytic contamination is one with which I am so thoroughly familiar that I should never fail to recognize it and to purify further any culture in case it existed. It would be impossible for me to mistake a mixed blackleg-proteolytic infection or a culture from such an infection for "malignant edema." I think that a great many mistakes may be traced to the fact that the blood of an infected animal usually does not contain enough organisms for the inoculation of animals or even for reliable colony work, and therefore transudates have been used for inoculation purposes. But if one take the heart blood of an animal dead from a blackleg infection or an infection by a member of the vibrion-septique group, inoculate it into a tube of meat medium, and incubate the tube anaerobically over night, one will invariably secure the principal invader, usually, but not always, in pure culture. It never pays to use subcutaneous serosity or peritoneal or pleural transudate for cultural purposes. One should beware of the fact that certain other types of anaerobes, notably B. oedematiens and B. Novyi, frequently kill guinea-pigs without becoming septicemic, and must be obtained from invaded muscle or from edema fluid; from undiagnosed infections, therefore, cultures should be made from various regions.

The critic will say, however, that, given a mixed culture of blackleg, vibrion septique, and a proteolytic organism, I might eliminate quite satisfactorily the proteolytic organism and lose also the blackleg strain because of the presence of the more highly pathogenic strain of the vibrion-septique group. I confess that this might happen and that it may have happened in the series of strains here presented. But if the blackleg strain and the vibrion-septique strain were present in abundance, as they usually are in material from an animal that they have killed, I should emphatically assert that they would both easily be detected in the guinea-pig, pathologically and microscopically. I know my critics will say that this is impossible, but not only am I entirely confident that it is possible, but I have three cases to prove it. I should remark first, that no culture ever passes my hands for inoculation purposes without microscopic examination of a Gram stain made from it; second, that careful necropsy examinations are made of all animals, their lesions noted, cultures taken from the heart blood and from infected muscle far from the site of inoculation, and smears or impressions made from the site of inoculation, inguinal region, axilla, peritoneal wall, liver surface, and from organs with unusual lesions. These smears are stained by Gram's method and carefully studied by myself. I have so studied every animal—over 200 in number—that has died of an anaerobic infection in this laboratory, and I should decline to accept the criticisms of one who was not in the habit of making like careful observations. Four times in the study of my material from cattle I have thought blackleg and an organism of the vibrion-septique group to be present in a guinea-pig infection. Three times I succeeded in isolating two strains—a blackleg strain and a strain of the vibrion-septique type—from the material. Each time I had thought blackleg to be present when examining the original culture which was used for inoculation. The fourth time a vibrion-septique strain alone was present (strain AS from a badly contaminated culture). It is an organism that forms spores lying sidewise in the bacillus and it forms large spoon-shaped orgonts; but otherwise it is to be assigned to the vibrion-septique group. I should hold that, had a cow died of a mixed vibrion-septique-blackleg infection, and were the two organisms present in anything like equal proportions in the material given me, I should hardly fail to detect both infections.

But my critics will say: "Suppose a cow died of blackleg and an organism of the vibrion-septique group contaminated the material—would you then not be likely to assign your case to mixed infection when the infection was truly monospecific?" The critics would be right. But the interpretation to be made of such a possibility should be on the basis of a true understanding of the chances of such a coincidence. It would probably be somewhat more likely that a vibrion-septique strain might contaminate blackleg material than that blackleg should contaminate vibrion-septique material. But the members of the vibrion-septique group are by no means ubiquitous in their distribution. Carl (c, 869) reviews this subject. Cornevin found the vibrion-septique type of organism rare in forest earth. Jensen found malignant edema rare in earth from various sources in the vicinity of Copenhagen. Feser infected only 7 of 30 rabbits inoculated with earth from various filthy places; among his samples was earth from the vicinity of anthrax cadavers. He does not tell us how many of his invasions were real infections of the vibrion-septique type. Carl concludes that the "malignant edema bacillus," under which name probably are included strains which are not of the vibrion-septique group, is widely distributed, but that it must not be regarded as a ubiquitous organism. We must also remember that we have no proof that the blackleg type of organism is not almost as abundant in distribution as are the organisms of the vibrion-septique group. The blackleg organisms are not pathogenic for many animals, but both types are highly pathogenic for cattle, and cattle are frequently attacked by blackleg. It is not even proved that blackleg organisms are regional in their distribution because blackleg is usually a regional disease. It is more than probable that some factor other than the mere presence of the organism induces infection, and it may be that such factors, rather than the bacillus, are regional in occurrence.

Another point indicates that my strains are not to be taken as grossly misrepresenting the flora of cattle and sheep infections. Guinea-pigs are subject to invasion by various soil organisms; yet I have found no strain from cattle material which was capable of invading guinea-pig tissue on its own initiative which does not fall into one of the two classic groups of cattle invaders. The nonsporulating tissue invaders are, I admit, to some extent eliminated by my technic, but nevertheless, had specifically abundant and gross contaminations of my material taken place before it reached me, other organisms would have been found, as one finds them any day in soil, which would have been pathogenic for guinea-pigs.

As to the material which came into my hands in the form of cultures: For the original mode of isolation of this I can take no responsibility. I place particular importance on that derived from cattle because it was called blackleg by someone else, presumably by the veterinarian who diagnosed the case in the original animal or by the bacteriologist who first studied the organism. It is possible that in one or two of my cases an original blackleg may have been supplanted by a vibrion-septique organism, but to my mind it is improbable that it happened in many cases.

4. STRAINS ISOLATED FROM CATTLE MATERIAL

From cattle material there were isolated 19 strains of anaerobes that are referable to the blackleg group and 13 strains referable to the vibrion-septique group. Three mixed blackleg-vibrion-septique infections were recognized.

Though I regard these findings as of great importance, I do not wish to be understood to present these figures as exactly representative of the relative frequency of these infections in cattle. There are several points that make them ineligible as statistics.

First, they are insufficient in number and they represent scattering cases from the United States and a few from Europe.

Second, their history is varied and inaccessible to me.

Third, some of them have been selectively weeded out by persons cognizant of the difference between blackleg and vibrion-septique infection—Eichorn's strains which he used for vaccine, for example, were all five referable to the blackleg group.

Fourth, a worker would be somewhat more likely to fail to recognize the presence of a blackleg strain than that of a vibrion-septique strain in an old and partially run out culture, because B. Chauvoei is a less active grower and less pathogenic for the guinea-pig than the organisms of the vibrion-septique group. Likewise, blackleg would probably more frequently be entirely lost from cultures because of its delicacy.

Fifth, there is probably a greater chance of contamination of cultures and material by vibrion-septique organisms than by organisms of the blackleg group. This element, though it exists, is not, to my mind, of great importance. I hold that such contamination may have taken place in my material, but that it is unlikely that it often did so. A prime point of evidence in support of this opinion is the fact that the four mixed infections (one from sheep), recognized by me, came from original animal material, and the fact that less than a third of the older cultures contained a pathogen. Were contaminations an important source of vibrion-septique strains, the older cultures would contain a large number of such strains.

The agar and gelatin cultures may in general be taken to have passed through more inoculations and generations than the brain cultures, which were handled by workers more familiar with anaerobes than those who used agar and gelatin cultures. It will be noted that the handling and transplanting of anaerobe cultures, a process which tends always to promote contamination of such cultures, tends also greatly to weed out pathogens and to replace them with nonpathogens. This finding is fully substantiated by a study of human material and cultures from human material. The pathogens may be regarded as rare and as infrequent contaminators of other material. I have only once discovered a vibrion-septique strain as a contaminator of a culture when there was no record of the presence of an invading pathogen. The culture was a stock tetanus strain of whose previous history I am ignorant. I do not think that loss of virulence is to be considered in connection with these figures. Vibrion-septique strains apparently do not lose virulence; they are among the most highly pathogenic of organisms. It will be noted that strains of the blackleg group preponderate greatly over the vibrion-septique strains in original material from cattle. This might be taken as an indication of contamination of cultures by vibrion-septique strains when they are subject to laboratory processes; but it must be borne in mind that the majority of the muscle samples received were from laboratories that used the strains for the making of vaccines, and they thus were subject to a deliberate weeding-out process. That in old cultures vibrion septique should be the most frequent form may or may not be wholly due to the fact that B. Chauvoei is more delicate and more easily lost.

The following table may be of interest:

TABLE 2

STRAINS ISOLATED FROM THE CATTLE MATERIAL STUDIED

	Animal Material, 14 Samples	Brain Cultures, 7 Samples	Agar or Gelatin Cultures, 23 Samples
2 pathogens isolated	3	0	0
Blackleg group	3		
Vibrion-septique group	3		
1 pathogen isolated	11	7	7
Blackleg group	11	3	2
Vibrion-septique group	0	4	5
No pathogen isolated	0	0	16

I must, however, say frankly that I should hesitate to conclude from the above figures and to assert emphatically that vibrion-septique infections were common in cattle, were it not for the fact that I feel that the accounts in the literature support me in such a conclusion, and that the close analogy between the infections of cattle and those of horses and sheep and hogs points to this probability. Countless evidences in the literature bear me out in my contention that strains of the vibrion-septique group are frequently isolated and identified as blackleg. Again and again one finds them—many have been quoted in this paper and many more will be observed on more careful search. Examples of these mistakes may be found with like frequency in various countries, and the errors have been made both by pathologists and by trained bacteriologists. They were made many years ago and they are made today. Only a few workers, all of them men who have themselves handled numbers of strains, have seen the light on this subject. Some of them worked when bacteriology was young and some have worked more recently. They express themselves, their work becomes classic, and then it is promptly disregarded in its principal points by the rank and file.

I feel justified in charging the majority of the members of the veterinary and bacteriologic professions who have dealt with the subject, with having misunderstood the etiology of the anaerobic invasions of cattle. They must either give up the habit of assuming that the lesions hitherto diagnosed as "blackleg," or considered as characteristic of blackleg, may be so diagnosed without differential bacteriologic examination; or they must completely relinquish, in connection with the conceptions "Rauschbrand," "symptomatic anthrax," and "blackleg," any idea that these names describe a disease sui generis, and must deliberately include under these names infections by the members of the vibrion-septique group.

5. STRAINS ISOLATED FROM SHEEP MATERIAL

I have isolated from sheep material two strains of organisms that are referable to the blackleg group. Both appear to have been the only pathogen present in their respective specimens. One, a strain apparently resembling in all respects the majority of those from cattle, was isolated from sheep material sent me by Dr. Hadleigh Marsh of Montana. He had diagnosed the case as blackleg. The other, from Halle, Germany, was derived from bradsot material sent me by Dr. Jensen; it is definitely referable to the blackleg group, but in morphology and in colony formation it differs from all the other strains under observation.

How much light my investigation of bradsot material will throw on the subject of sheep infections I do not know. The material studied was all from cases that had been diagnosed as "Bradsot" by different workers in different countries. It is therefore parallel to my cattle material which had all at some time been diagnosed as "blackleg." I have to thank Dr. C. O. Jensen of Copenhagen for all the samples studied. They were, with one exception, pieces of muscle from what I understand to have been the original sheep. There were 11 samples: from 3 I could isolate no pathogen, from 1 the blackleg-group strain RG was isolated, and from 7 samples strains of the vibrion-septique group were isolated. One specimen from Sletten, Norway, furnished two species of vibrion-septique type. They differ markedly in colony formation and in pathogenic action on the guinea-pig, one producing on thigh inoculation the most marked gastritis observed in this laboratory, with a bright red coloration over the whole external stomach surface; the other producing a relatively small amount of injection in the abdominal cavity. These 8 strains are all definitely to be included in the vibrion-septique group as defined on page 5, b. They are by no means all of the same species, but show on careful study various differential characters. A definition of these characters is forthcoming. If these strains are to be accepted as the etiologic agents in cases of Bradsot, then we must say that Bradsot is not a disease sui generis

I realize fully that my lack of case histories must qualify any statement as to the causative rôle that these organisms played in the death of the sheep from which they came. The final criteria to be used in judging of what is braxy and what is not braxy, of what constitutes anaerobic infection and of what must be ruled out as not constituting anaerobic infection are, to my mind, still to be established. But the fundamental bases for such criteria should be etiologic and histopathologic ones.

The fact that pathogenic anaerobes may be found in animals dying of other causes is, to my mind, entirely beside the question. Careful and intelligent determination of the quantity and specific nature of the anaerobic invaders in fresh cadavers of sheep showing "bradsot" lesions and in fresh cadavers of sheep dying of other causes is the only way to make observations that can be considered of great value.

Data concerning other strains isolated will be found in the next part of this article.

A note as to the subdivision of the groups discussed is perhaps in order. Various authors have noted differences in anaerobes which had been classified as one species by other workers. Many of these differentiations depended on culture-contaminations. Under this head I should classify Titze and Weichel's strains, Markoff's proteolytic and nonproteolytic chain-forming strains, and Klose's strains. Some divisions depended on an association and further division of unrelated organisms. Kirsten investigated many carefully-isolated strains of what he calls the edema bacillus. He found the organisms in feces and in the blood of various animals. Most of them were nonpathogenic. Kirsten describes 10 types which can be distinguished by morphology and by cultural characters. I interpret these strains to have been members of various saprophytic and parasitic groups. Some differentiations have been made whose value I am unable to determine. Poels, whose original article is inaccessible to me, cultivated blackleg bacilli from what I take to have been the inoculated thigh of guinea-pigs. He noted differences in the gas formation, in the time of sporulation, and in the pathogenicity of his strains. Grosso divides his blackleg strains into "Rauschbrand"—nonpathogenic for mice—and "Pseudo-rauschbrand"—pathogenic for mice. Robertson (Med. Res. Comm. 39, 53) has divided vibrion septique into 3 groups on the basis of the agglutination reaction. Weinberg and Séguin (b) note minor differences in their vibrion-septique strains.

A few details as to the differences between the strains in my collection must be included. Most of the blackleg strains behave alike, so far as they have been studied. These I call temporarily "typical" blackleg. They form heavy citron-shaped orgonts, are quite highly pathogenic for guinea-pigs and are not pathogenic for rabbits. Their colonies are similar. A few other strains behave differently. One is different in morphology and is only slightly pathogenic for guinea-pigs. Another has never been observed to form citron-shaped orgonts, another forms colonies that are unique. A careful investigation of other characters of these organisms is in progress. Until this investigation is finished I must decline to divide the group in a dogmatic manner. The strains of the vibrion-septique group are divisible into subgroups by agglutination, by colony formation, and to some extent by morphology. I must also decline to arrange these divisions for the present.

III. THE ANAEROBIC INFECTIONS OF ANIMALS AS RECORDED IN THE LITERATURE: A CRITICAL ANALYSIS

(*a*) *The History of Cattle Infections Up to* 1905.—This article deals with the organisms involved in the anaerobic infections of animals. I am in no way prepared to discuss with authority the subject of the pathology of the disease of blackleg in cattle, and must be content to refer the reader to some of the notable works on that subject. It will be convenient to discuss simultaneously the two types of organisms and the diseases caused by them.

Anthrax and symptomatic anthrax (blackleg) are two diseases that are in general so similar that ancient and medieval authors do not distinguish them. According to Arloing, Cornevin, and Thomas, who have most carefully reviewed the literature from ancient times on, a satisfactory and logical classification of anthracoid diseases was not made till Chabert undertook to arrange them according to clinical symptoms in 1782. He classified them thus:

Fievre charbonneuse: "When the disease develops without manifesting its existence by external tumors, it is anthrax fever or internal anthrax."

When tumors appear, it is "essentiel" or "symptomatique."

Charbon essentiel: "It is "essentiel," when the tumor begins at the onset of the disease without former symptoms and without signs of sickness except those which result from its existence, the tumor being at first small, hard, renitent, painful; then enlarging, and only after enlarging producing grave constitutional symptoms. When incised these tissues are black, like gangrenous tissues."

Charbon symptomatique: "It is symptomatic, when the tumor follows a rise of temperature, loss of appetite, arrest of digestion, chills, and rigidity."

Feser is given credit for being the first to distinguish Rauschbrand from anthrax in recent times (1876).

Bollinger was the next to publish material on the subject (1878).

Arloing, Cornevin, and Thomas, in 1879 and following years published a series of papers concerning the disease, which afterward were condensed in book form under the title "Le charbon symptomatique du boeuf" and appeared in two editions, the latter in 1887. They reviewed all the literature available on the subject and published accounts of their own valuable researches.

As to the distribution of the disease they summarize: "In a word, wherever the dominant branch of agricultural industry is the raising of cattle . . . symptomatic anthrax is to be found." They note that in Switzerland there is approximately ten times as much symptomatic anthrax as true anthrax. The disease occurs throughout the year, but is far more frequent in June, July, August and September than in the other months. Moisture and a high temperature coincident with a rapid multiplication of the organisms is conducive to increase of the disease, and the geologic substratum of a region has nothing to do with it. The largest percentage of victims are animals between 6 months and 2 years of age; young calves are immune but later become susceptible to the disease. The animals attacked are usually in excellent condition, and males are apparently more often attacked than females. The right side of an animal is more often attacked than the left. The disease is almost invariably fatal. Arloing, Cornevin, and Thomas identify both the "charbon essentiel" and "charbon symptomatique" of Chabert as symptomatic anthrax and not as anthrax. They describe the disease as an emphysematous affection of the muscles. On incision of a tumor the muscles may be colored dark like wine dregs toward the center, and red, pink, or yellowish toward the periphery.

Blackish streaks permeate the lighter portions. Around the tumor in loose connective tissue much edema is found. Near the affected muscles this is red and contains filaments of fibrin. Farther away it is colorless or slightly yellowish, and very mobile. When the edema is abundant the gaseous infiltration is less, and vice versa. In the guinea-pig gas formation may be absent. Many other pathologic details are given.

The organism causative of the disease is found in great numbers in the affected muscles, in the serous exudate surrounding them, and in the affected organs; and it is found in the blood. Three forms of the organism are found in the blood and in the edema fluid near the muscle tumor:

1. Micrococci, often very pale, difficult to see in the plasma. Their presence is revealed principally by the movements which they communicate to the red cells; they are more easily placed in evidence by the aid of anilin blue or vesuvin; they measure about 0.0002 mm. in diameter (0.2 mikron).

2. Long bacteria, 0.005 mm.—0.008 mm. in length and 0.001 mm. in thickness (5-8x1 mikrons), which are highly motile; they rise and descend in the liquid of the preparation, bend in the form of an arc or half circle, and pirouette, lying flat, oblique, or on one end.

3. Nucleated bacteria, 0.005-0.01 mm. long and 0.0011-0.0013 mm. wide 5-10 x 1-1.3 mikrons); a brilliant corpuscle occupies about a third of the length. They are less motile than the bacterium (form 2), oscillate, but do not bend. When an organism is long it sometimes contains a corpuscle at each end and is articulated in the middle. These types may be thickened at the portion containing the spore and may be fusiform.

The organism does not take anilin dyes readily. Arloing, Cornevin, and Thomas give three lithographs of the bacilli in the unstained condition, but do not represent the micrococci.

After careful consideration of systematic details, the authors named their organism Bacterium Chauvoei (not Chauvaei and not Chauveaui).

In practically every detail, the organisms that I have identified as the organisms causative of blackleg correspond with the description given by Arloing, Cornevin, and Thomas and with their lithographs. The micrococci I have not discovered, nor have I found other references to them. It is probable that they were not spores, for spores are oval and at least 0.5 in diameter. From the statement as to anilin blue and vesuvin it seems as though they took stains: whether they were blood platelets, micrococci sui generis, débris, or slender bacilli on end cannot, perhaps, be determined.

Arloing, Cornevin, and Thomas declare their organism to be pathogenic for cattle, sheep, and guinea-pigs, usually also for goats. It produces local processes in the white rat, the ass, and the horse, but is not fatal to them. The hog, dog, cat, sewer rat, duck, fowl, and pigeon appear to be entirely unsusceptible to the disease.

Intravenous and pulmonary injections of virus are ineffective. Inoculation must be made subcutaneously or intramuscularly. Animals cannot be infected by a scratch with a lancet, or by the introduction of virus into the pulmonary passages. The authors tried many times to infect an animal by feeding and succeeded once only, by feeding a bull calf bread soaked in extract from a guinea-pig which had just died of blackleg. Intravenous inoculation of large doses infects only occasionally, producing internal lesions and no skeletal lesions. The authors believed the protective mechanism of animals against blackleg to be phagocytic, but thought also that the endothelial cells might act as a filter to the virus, because animals infected by intravenous inoculation and then wounded in the skeletal muscles develop a typical tumor at the site of the wound.

Arloing, Cornevin, and Thomas found that the bile of stricken animals is rich in specific bacteria, the amniotic fluid is also heavily infected, the muscle pulp of the blackleg tumor is very rich in organisms, the edema fluid about the tumor varies in its bacillary content, the blood contains the organisms, but only late in the course of the disease, the aqueous and vitreous humors are very slightly virulent, and the urine is never found to be infective.

The authors found the organisms difficult to cultivate, but apparently succeeded with chicken broth to which a little glycerol and iron sulphate had been added. They incubated the cultures under CO_2, and raised successive generations of an organism. The cultures increased in virulence and killed the guinea-pigs in a decreasing period of time. The animals that were killed by the later generations of culture showed only a serous edema and bacteria that were clove shaped. The authors doubted the purity of the culture, but convinced themselves that the organism was not a vibrion septique (organism of septicémie foudroyante). Whatever the organism was, it can hardly have been blackleg.

The authors showed by primitive methods (filtration through plaster, or by allowing the bacilli to settle) that the filtrate from blackleg lesions does not communicate the disease.

In a detailed fashion they brought forward many differences between their disease and anthrax. They were familiar with the vibrion-septique type (Chauveau's organism of gas gangrene), and distinguished it from the blackleg bacillus by its filament formation. They distinguished their disease also from the septicemias of Davaine, of Pasteur, and of Koch.

Ehlers investigated the cultural behavior of the organism in 1887.

Kitt (b) was probably the second to compare B. Chauvoei with the organisms of the vibrion-septique type. He pointed out the great similarity between them, inoculated both types into cattle, and noted the long chains of the "malignant-edema bacillus" as the only marked distinguishing factor.

W. Koch stated that edema bacilli may accompany blackleg bacilli into the tissues of the affected animal.

Kitt (c) reviewed the subject to date, adding accounts of his own researches, and his article has become a classic in the field. He went into considerable detail concerning the natural mode of infection and protective inoculation against the disease.

Pasteur in the late seventies had failed to cultivate his vibrion septiques in various mediums in the presence of air; he then grew them in broth which was incubated anaerobically. Roux, in 1887, grew them on solid mediums and described the colonies.

Roux (a) grew the bacillus of symptomatic anthrax in liquid mediums in an anaerobic apparatus. The photograph given of the organism in the muscle of the guinea-pig is, however, evidently of a member of the vibrion-septique group.

In 1889, Kitasato described the cultural characters of the Rauschbrand bacillus. He was clear in his differentiation of the organism from that of malignant edema, stating that the Rauschbrand bacillus is slender and does not grow out to long threads; he identified the short type with that described by Arloing, Cornevin, and Thomas. Kitasato did not credit the cultural work of Ehlers and of Koch. He grew the blackleg organism in guinea-pig broth and found that it clouded the broth, then formed "Flöckchen" (auto-agglutination?) and settled. The odor was that of rancid butter. Kitasato stated that he was unable to grow the organism in pure culture on solid mediums. He purified it by a dilution method and declared it to be a strict anaerobe that would not grow under CO_2. He distinguished it culturally from the bacillus of malignant edema.

In 1890, Kitasato described a method of growing Rauschbrand on solid mediums. He studied the biology of the organism further, showing that the shining bodies, by which he probably meant the chromophyl bodies, were not resistant forms; the spores he found present in the animal body only some time after death.

In 1900 Le Clainche and Vallée presented immunologic work of distinct value. They distinguished sharply between B. Chauvoei and vibrion septique, using the formation of filaments as the principal differential criterion, saying in the earlier paper that rabbit inoculation was differential, but in the later paper stating that B. Chauvoei may at times kill rabbits. Their biologic studies showed that a toxin is produced by the organism in cultures of Martin's broth and that it can cause grave lesions and even death. They showed also that the washed spores of the organism, when inoculated in large doses, are incapable of infecting animals, and that the resistance of the animal to infection lies in its power of phagocytosis.

Schattenfroh and Grassberger came forward in 1901 (a) with their identification of Rauschbrand with the bacillus of Fraenkel. They had not previously worked with Rauschbrand, and on finding Fraenkel's bacillus (unbeweglicher Buttersäurebacillus) in various samples of Rauschbrand material they jumped to the conclusion that Fraenkel's bacillus was the cause of the disease. They were so impressed with this finding that they burst into print a few days later and they also attacked all their predecessors in the field, including the careful Kitasato, for having worked with mixed cultures. This was probably in some cases justifiable, for Schattenfroh and Grassberger found some cultures of Rauschbrand to be impure. But their identification of the causative organism as Fraenkel's bacillus was made without study of the pathologic processes involved, and was as superficial a piece of work as ever found its way into the literature.

Schattenfroh and Grassberger emphasize continually the necessity of working with pure cultures. They themselves isolated anaerobes by a plate colony method. Still, one sees throughout their work continual glimpses of the fact that their critique in this matter was insufficient. In the article on malignant edema (b) they narrate their experience with four strains of malignant edema, which they characterize as a putrefactive organism, and yet two of the four strains probably came into their hands in a pure condition because they were the vibrion septique of Pasteur, sent by Metchnikoff to Schattenfroh and Grassberger, and the gas phlegmon bacillus isolated that spring from man by Ghon and Sachs, and sent by them to Schattenfroh and Grassberger and described by Ghon and Sachs as nonproteolytic.

A few months after their first publication on the subject of Rauschbrand, having worked a little more extensively with Rauschbrand material, and having several times encountered the true Rauschbrand organisms face to face, Schattenfroh and Grassberger volunteered (*Münch. med. Wchnschr.*, Aug., 1901), a theory which in its later development was destined to cast on anaerobic studies a depressing blight, which has lasted until today. Having found Fraenkel's bacillus in Rauschbrand material, and having told the world of their great discovery, namely, that it was the true cause of Rauschbrand, they were loth to grant to the form whose acquaintance they later made any specific identity. But this form evidently was the one described by other workers (it was motile and also pathogenic) and so they were obliged to give it recognition. They built up the thesis, for which they are today known, on the study of the behavior of mixed cultures of these two forms.

Their conclusions in August, 1901, were: The Rauschbrand bacillus is a butyric-acid ferment producer which forms granulose when it sporulates. Its development passes through a double life cycle: One phase is flagellate, motile, forms granulose, sporulates, and is difficult to isolate. The other phase is aflagellate, nonmotile, does not sporulate, and forms colonies exactly like the "unbeweglicher Buttersäurebacillus." Phase one is highly pathogenic and produces hemorrhagic edema and little gas in the guinea-pig, and a soluble toxin in culture mediums. Phase two is less pathogenic and less of a toxin former. It produces in the guinea-pig the typical picture of gas phlegmon or that of malignant edema. These types may be changed one into the other by growing them on various mediums: the nonmotile form is likely to supersede completely the sporulating form.

Grassberger (Anpassung und Vererbung bei Bakterien) explains the process of denaturing Rauschbrand bacilli. He says that if the Rauschbrand organism, which is a motile, nonproteolytic, sporulating anaerobe, is rapidly passed through sugar containing mediums before spore formation takes place, it becomes denatured; that is, it loses its flagella and becomes chunky, and is then identical with the most common organism that causes human gas-gangrene—the nonmotile butyric-acid bacillus (B. Welchii). But if the Rauschbrand bacillus is carefully grown successively on sugar-free mediums, it becomes proteolytic, the spore moves to one end, and is sharply differentiated from the bacillus; the organism remains motile, and resembles Bienstock's organism. Grassberger was also able to change the Rauschbrand bacillus to an aerobe by surface cultivation. In fact, the photomicrographs show that it grows far more rankly as an aerobe than as an anaerobe. Efforts to make an anthrax bacillus out of a Rauschbrand bacillus arrived so far as to produce "schnörrkeln." The Rauschbrand bacillus was changed into ten different types of organisms, but it always (except in the case of the first type mentioned?) lost its pathogenicity by such changes.

It is impossible for any one who has critically handled anaerobes to give the results of Grassberger the slightest credence. In such a matter as this the judgment of experienced workers must be the deciding criterion. Definite proof of radical changes in the behavior of organisms, which is controlled by detailed accounts of the technic used in the isolation and in the study of those organisms and is substantiated by repeated evidence, is necessary to produce conviction. It is most easily conceivable that the sterilizing apparatus in the laboratory of Grassberger and Schattenfroh was an unusually inefficient one, but such changes as they describe occur in the best regulated laboratories, and critical examination of cultures whose habits have changed always reveals the presence of two or more forms, which when isolated behave consistently through many generations and on a great variety of mediums.

I insist that the work of Schattenfroh and Grassberger has proved of great detriment to their branch of bacteriologic science. Its blighting effect has been noticeable in textbooks and in many laboratories. This blight is effective in a simple manner: If anaerobes may change their nature the investigation of their characters with a view to classification is futile. The extreme effect of this doctrine is to be noted in the headlong work of Conradi and Bieling, who declare one single species of anaerobe to be the cause of gas gangrene. This organism, B. sarcemphysematodes hominis, possesses, according to descriptions, the characters of all the anaerobes with which we are familiar. I possess a culture of it which I received through the kindness of Dr. J. L. Burckhardt, who obtained it from Kral's collection. It is now entirely nonpathogenic and highly proteolytic, containing chiefly the sporogenes type of organism. W. Kolle, Ritz, and Schlossberger studied anaerobes extensively, accepted the work of Grassberger and Schattenfroh and interpreted their contaminations in the light of it, declaring that no species is well defined or definitely distinguishable from others.

Men who have worked with anaerobes and studied them creditably have also accepted the theory of Grassberger and Schattenfroh. Because of his acceptance of this theory, Foth refrains from studying closely his chain-forming organisms. Ghon, anno domini 1917 says: "Die Anschauung von Grassberger und Schattenfroh so wie Conradi und Bieling über die Möglichkeit, anscheinend unbewegliche Bazillen vom Typus des Fraenkelnschen Bazillus in bewegliche unzuwandeln womit auch anscheinend neue kulturelle Eigenschäften gewonnen werden, ist zweifellos richtig." I can only reply to this that after long acquaintance with the habits of anaerobes, I am entirely unconvinced of the occurrence of any such changes.

(*b*) *Vibrion Septique and Atypical Blackleg Group Infections of Cattle.*—Malignant edema has often been reported in cattle. I have neglected to collate as long a list of such reports as I might have done, and I am ignorant of the period in which such infections were first recognized.

In 1895 several authors reported malignant edema in cattle, which usually followed parturition or wounds. Home, Attinger, and Reuter made such reports.

Wankmüller reports two cases of malignant edema in cattle, in which no portal of entry was demonstrable.

Von Hibler (a, 404) states that he isolated the bacillus of Ghon and Sachs from a cow that died some days after aborting. There seems, however, to be no definite indication that the case was one of uterine infection. The cow showed edema and emphysema of the subcutis of the right shoulder and moist hemorrhagic muscle beneath. Peritoneal and pleural linings were somewhat reddened and both cavities contained transudate. No statement is made as to how the shoulder infection originated.

Mohler states that the malignant edema organism has been obtained in his laboratory from the infected muscles of a calf that was supposed to have died of blackleg. Therefore all blackleg virus made in the laboratory is thoroughly tested before it is made into blackleg vaccine in order to exclude the malignant edema organism.

Puerperal blackleg has often been discussed. Dr. Meyer informs me that some cases of so-called puerperal blackleg are not due to anaerobic invasion. Many cases have, however, been reported as due to organisms of the vibrion septique group.

Albrecht reports cases of puerperal Rauschbrand which were caused by organisms of the malignant edema group, and he gives the symptoms and pathologic changes caused by the disease.

Strebel can see no reason for distinguishing puerperal Rauschbrand from true Rauschbrand on clinical grounds, and he compares the two diseases clinically.

Carl (b) identified the bacillus of malignant edema as the cause of 3 cases of puerperal Rauschbrand.

Markoff, in Kitt's laboratory, investigated with much care, the organisms found in 4 cases of puerperal Rauschbrand. From 1 he isolated a strain of true blackleg, from 2 cases he isolated the nonproteolytic chain formers D and E, and from 1 case he isolated a proteolytic chain former which he calls malignant edema.

Foth (a, 206) reports from his own observation 2 cases that followed calving. In 1 the muscle was not seriously attacked and was very moist. The invader was a chain forming organism. In the other case a cow died with typical Rauschbrand 3 days after calving and the invading organism was true Rauschbrand.

Wulff (42) states that he has studied 3 cases of puerperal Rauschbrand. The muscle sent to the laboratory was exactly similar to that of animals dying of true Rauschbrand, but the bacillus of malignant edema was the invader isolated.

I think that the conclusion of Markoff may be accepted: Puerperal Rauschbrand is not a specific disease but may be caused by a group of related anaerobes.

Oppermann describes carefully cases of malignant edema in sheep after parturition.

Some reports of unusual blackleg infections cannot be accurately placed.

Schöberl describes a case of primary intestinal Rauschbrand. He attributes the infection to the fact that the cow had to eat dirty food.

Tillmann gives certain clinical notes regarding Rauschbrand. He notes 2 cases in which only the inner organs were affected.

Laabs describes 2 cases of cattle infection that he calls Rauschbrand. Muscular lesions were entirely absent in both cases. The tissue involved in the first case was the connective tissue in the region of the udder, the intestinal mucosa, which were injected, and the spleen, liver, lungs, heart, and pharyngeal region. In the second case the connective tissue of the region about the udder and under side of the thigh was highly edematous and emphysematous. The mucosa of the vagina and rectum were dark red. There was peritoneal transudate, and the liver, spleen, lungs, and heart were also affected. Laabs carefully searched the textbooks for differential characters between malignant edema and blackleg; he found many such criteria and studied the organisms from his 2 cases according to them. He diagnosed the disease as true blackleg. I cannot agree with Laabs in his diagnosis, but must lay the blame for his conclusions on the textbooks which he so carefully followed. His criteria were either absolutely wrong, unessential or misleading. Also, in looking for filaments in the animal body I think that Laabs failed to look in the peritoneal cavity, except in the case of a guinea-pig that he inoculated from the liver of one of the cattle—this guinea-pig he stated had died of malignant edema. Laabs' organisms form abundant visible colonies in agar inside of 24 hours, and they form many median and subterminal spores. They coagulate milk in 2 days, and they produce abundant gas in the subcutaneous tissue of the guinea-pig. None of these characters are easily referable to blackleg.

Kiesel reports Rauschbrand cases in which the infection was apparently localized in the heart muscle and the spleen; and the small intestine, which showed erosions, was much inflamed.

Foth (a, 205) had never seen cases like those of Tillmann and Kiesel, but he had seen cases in which the muscle lesion is small, moist, and blackish red.

Wulff deals extensively with this subject. He found, in a long series of cases, 6 that showed little muscle affection and 9 that showed no muscle affection. His necropsy examinations were carefully made, and he investigated nearly all of the cases bacteriologically. In each case that he investigated the invading organism was shown to be true Rauschbrand.

K. F. Meyer (c) describes 2 cases of blackleg in cattle in which muscle lesions were absent and in which liver infarcts were present. B. Chauvoei was isolated from both cases.

As stated before, I am in no position to express an opinion on the pathology of these diseases in cattle. I think that certain suggestions are in harmony with those of Foth and others, and that on the analogy of the behavior of these diseases in other animals certain lesions will be found characteristic in cattle. Blackleg organisms show in general a preference for skeletal and heart muscle, and they produce a greater amount of hemolysis and a smaller quantity of edema, gas, and transudate than do those of the vibrion septique group. Gas is probably most frequently in the form of minute bubbles in the muscles themselves. The organisms of the vibrion septique group grow well enough in muscle but will probably be found to kill, in nature, most often before muscle invasion is extensive. They prefer areolar tissue and mucous membranes and serous linings to muscle. Muscle lesions when present probably do not show the dry, spongy, almost brittle character of blackleg muscle lesions and may be markedly infiltrated. The organisms of the vibrion septique group produce abundant gas in areolar tissue and in muscle, where it is in larger bubbles than in blackleg lesions, and where it is interstitial in its distribution, and they produce little or no gas in the mucosa. The type of infection, apparently rare, in which the mucosa of the alimentary canal are markedly injected may well be termed "braxy" or "Bradsot" of cattle.

(c) *The Recent History of the Infections of Cattle.*—Gutzeit and Carl (a) presented points of difference between Rauschbrand and malignant edema. Waringsholz gives clinical contributions. Their three papers are inaccessible to me.

Perhaps the next work of importance is that of Foth (a and b). Foth worked in Schleswig, where Rauschbrand is abundant. The object of his paper is to make it possible for the veterinary pathologist to say with some certainty what is Rauschbrand and what is not Rauschbrand. Foth's understanding of Rauschbrand is excellent. It is probable that in using carefully the criteria given us by him one would never go astray in the diagnosis of pure infections.

He gives us a detailed account of the pathologic lesions which in cattle may be considered as diagnostic of blackleg. He also gives us in detail an account of lesions which must be regarded as possibly indicative of blackleg. In such cases he advises the use of the guinea-pig as an aid to accurate diagnosis. Foth regards as of prime importance the formation of filaments on the liver of the guinea-pig by the usual type of invader that is not blackleg (verbandbildende Bakterien), and the absence of such filament formation by blackleg organisms; he also regards as of importance the injection of the serous lining of the peritoneal wall and the injection of the intestines of the guinea-pig in the case of the infections caused by chain forming bacteria; and the absence of such injection in the case of blackleg infection. He further advises investigation of the colony formation of the organisms found, and his account of the colony formation of these organisms agrees exactly with von Hibler's and with my own. I do not feel, however, that Foth lays enough stress on the deceitful rôle played in colony work by the usual contaminating organisms in anaerobe cultures. Let me also recommend to the interested reader the excellent photomicrographs of blackleg organisms given by Foth in volume 8.

There is, however, a weakness in the work of Foth that I may lay to two causes. First, the generally accepted conception of "chain forming" anaerobic bacteria was at Foth's time (1910) entirely wrong, and Foth's conception is in accord with that of others. Second, the law in Germany authorizes the payment of compensation for animals dead of Rauschbrand but not for those dead of infection by related organisms. Foth, therefore, contented himself with deciding what was blackleg and what was not blackleg, studying the Rauschbrand organism in some detail, and neglecting the other type of invader almost wholly. He does not tell us what percentage of the cattle infections that came under his observation were attributable to true blackleg and what percentage of blackleg-like infections were attributable to the chain-forming organisms. He observed blackleg in 346 guinea-pigs infected with various blackleg strains and observed infections by chain forming organisms in 190 guinea-pigs infected from various types of material. It is, however, implied in his work that "Rauschbrandähnliche" infections of cattle are by no means rare. Still, Foth is of the opinion that cattle infections that do not follow external wounds or parturition and which show characteristic Rauschbrand-like lesions of the skeletal muscle, may safely be diagnosed as Rauschbrand without a guinea-pig inoculation. I do not agree with him in this conclusion, though I am in no position to judge just which lesions in cattle may be truly characteristic of Rauschbrand and which may not. Neither is the veterinary pathologist who has not made a study of the subject qualified to state what is truly characteristic of Rauschbrand and what is not.

It appears to me that the possible pathogenesis of the "chain forming" organisms for cattle is much neglected by Foth. This is due to the fact that very few workers in 1910 had ever isolated such chain formers and studied them. Foth found them to be far more highly pathogenic for laboratory animals than blackleg strains were, but still he persists in regarding them in general as contaminations of blackleg material. He himself found these strains all to be more or less proteolytic, stating that none approached the bacillus of Ghon and Sachs in cultural characteristics. One is thus forced to charge him with never having isolated one of his chain forming organisms. Bound up inevitably with such a conception as Foth's is its corollary: that the "malignant edema" organisms are universal in their distribution, and pathogenic only when introduced into wounds.

But let us free ourselves from all prejudice and take a view of the relationships of these organisms that is based on a study of the pure strains. The organisms of the blackleg group and those of the vibrion septique group are culturally closely related, and the conditions governing the occurrence in nature of the one type cannot be very different from those governing the occurrence of the other type. The organisms of the vibrion septique group are probably somewhat more abundant in nature than those of the blackleg group because they grow somewhat more abundantly in artificial mediums than do those of the blackleg group. But they are by no means universal proteolytic cadaver and putrefactive bacilli. The organisms of the vibrion septique group are, however, far more highly pathogenic and more generally pathogenic for vertebrates than are those of the blackleg group. Those of the blackleg group are highly pathogenic as a spontaneous infection for cattle and sheep only, so far as we know. But that, in the face of this unusual susceptibility to blackleg shown by cattle, one should conclude that cattle are not to be considered readily susceptible to organisms that produce lesions so like blackleg lesions as do those of the vibrion septique group, is to me a glaring inconsistency. I do not by any means intend to say this in arraignment of Foth alone. Other careful workers have made the same mistake.

This should be emphasized: When a worker with suspected blackleg material from a cow finds both true blackleg and an organism of the vibrion septique group present in the affected muscle of that cow, he has no right to conclude that the organism of the blackleg group alone was the cause of death of the cow; and a worker who finds an organism of the vibrion septique group and no organism of the blackleg group in the affected muscle of a cow dead of a blackleg-like disease is as justified in pronouncing such a strain of the vibrion septique group to be the cause of the death of the cow as he would be justified in pronouncing the disease "blackleg," were the organism of the blackleg group. That organisms of the vibrion septique group are pathogenic for cattle needs today no further proof. Why then persist in overlooking such infections?

The serious work of Wulff is to be recommended. He neglects, however, the subject of the chain forming bacteria. It is evident from the work of Foth and of Wulff that the anaerobic infection in the region about their Schleswig laboratory is largely true blackleg infection, but I do not think that we have reports from other districts that are accurate enough for us to decide what the relative proportions of these infections are.

(*c*) *An Oedematiens Group Infection in a Cow.*—Kerry describes an organism of oedematiens type which he isolated from a cow that was thought to have died of blackleg. The organism, according to its description, resembles the B. oedematis maligni II, described by Novy in 1894 and isolated by him from a guinea-pig that had been inoculated with milk nuclein. Kerry did not work with Novy, as has often been stated. Whether exact cultural comparison of the two strains was ever made I am unable to determine. Kruse identifies the two strains and names them B. oedematis thermophilus Novy. Migula renamed the species B. Novyi, which name is probably the least likely to lead to confusion. As identifications by means of printed descriptions are unsound, we must let the specific affinities of Kerry's organism go undefined until a strain can be compared with that of Novy and with those of Weinberg. Kerry's organism is far more pathogenic for guinea-pigs than any of the strains of B. Novyi in my possession, and the lesions that it produces (Kitt, e) resemble more closely those produced by the oedematiens strain Joly.

(d) The Epidemiology of Anaerobic Cattle Infections.—Under the above title must be considered the contributory mechanic-traumatic factors leading to infection.

Blackleg as a Wound Infection: Most older authorities agree that blackleg is a wound infection, although frequently the wound may not be large enough to be demonstrated. There are many accounts of necropsies in which no external lesion was found. Therefore many authors have concluded that infection probably often occurs through the alimentary canal.

Hutyra and Marek say (42): "More frequently (than by wound contamination) the infection results from the consumption of infected feed or drinking water. Artificially it is successful in this way only in exceptional cases (Bollinger, Arloing), but the fact that in blackleg regions the intestinal canal as a rule contains blackleg bacilli (Le Clainche and Vallée), while lesions on the surface of the body are observed only in exceptional cases, and furthermore that crepitant areas are present in the internal organs, speaks in favor of the frequency of infection through the intestines. In the rare cases when such swellings originate in the region of the throat or in the wall of the esophagus, the infection is evidently brought about through the mucous membrane of the throat or the mouth."

Kitt (e, 296) remarks that alimentary infection probably occurs, but that it is nevertheless a wound infection.

In discussing cases of unlocalized blackleg (blackleg in which no muscles are involved) Wulff (626) says: "Der Rauschbrand war bereits als Septikämie von uns erkannt worden. . . . Die alte Anschauung, dass der Rauschbrand lediglich als eine Wundinfektionskrankheit aufzufassen sei, war bei ruhiger Betrachtung und Würdigung der Sectionergebnisse an der grossen Zahl von Rauschbrandfällen nicht mehr aufrecht zu erhalten.

"Bei den Hunderten von Rauschbrandsektionen konnten in keinem Falle Wunden oder Narben, die als Eingangspforte angesehen werden konnten, festgestellt werden. Berücksichtigt man ferner, das nur in den seltensten Fällen nur eine bestimmte Muskelgruppe erkrankt ist, dagegen in der Mehrzahl der Fälle mehrere von einander entfernt liegene betroffen sind, dass ferner nicht selten Muskeln verändert sind, wo eine Aussen-infektion garnicht möglich ist, wie Z. B. bei Erkrankung der Lunge, des Zwerchfelles oder der Psoasmuskeln, so dürfte doch wohl die Schlussfolgerung berechtigt sein, dass der Infektionsstoff zur Hauptsache mit der Nahrung aufgenommen wird."

Blackleg as an Alimentary Canal Infection: The idea permeates the literature that malignant edema is a wound infection due to much graver wounds than those to which blackleg is due. Textbooks all give the disease as a wound infection, never mentioning possible infection through the alimentary canal, though it is now recognized that braxy may infect sheep in this manner, at least occasionally (see p. 67).

Mohler says: "Unlike blackleg, this disease (malignant edema) never appears as an epizootic but in isolated cases. It may also be differentiated from the former by the history of a recent parturition or surgical operation, by the presence of an external injury at the site of the swelling, accompanied with a fetid liquid discharge, and gangrenous appearance of the tumefaction."

Dr. Meyer assures me that in Switzerland, at least, blackleg occurs sporadically in nonimmunized herds.

Zeissler (a, 108) recognizes three cases of true puerperal Rauschbrand: those of Foth, Markoff, and Eug. Fränkel.

I can only emphasize here what seems to me to be a self-evident fact: The etiologic agency of a disease like malignant edema or blackleg, which follows a wound infection, depends entirely on what particular organisms happen to get into the wound, and puerperal infections are to be classed as wound infections. There should be no reason why blackleg infection may not follow a large wound as easily as it does a small wound, nor why true blackleg may not occur as a puerperal infection. The chances are that infections following small wounds are more likely to be monospecific than those following large wounds. The exposure to contamination is less in the case of small wounds and the resistance to strains of low pathogenicity is likely to be greater than in the case of large wounds. In the case of large wounds proteolytic organisms are more likely to invade along with the nonproteolytic ones, and to produce the mixed infections which, on account of their putrid odor, have so often been diagnosed as "malignant edema." It is possible that true blackleg may occur more frequently in the absence of macroscopic wounds and the other type may occur oftener when a wound is demonstrable. This may be due to a factor of bacterial distribution, or it may be due to some special invasive power of the blackleg organisms in the alimentary canal. I am, however, of the opinion that many mixed blackleg-proteolytic and blackleg vibrion-septique infections have probably been diagnosed as "malignant edema," and there can be no doubt that many pure vibrion septique infections have been diagnosed as blackleg (see p. 35). I think, therefore, that until this matter has been decided by bacteriologists we are not warranted in stating what organisms are principally involved in the different types of diseases.

Hutyra and Marek (38) state: "Malignant oedema is indicated by the appearance of the disease in localities where blackleg is not prevalent, further by the advanced age of the patient."

Friedberger and Fröhner say (328): "Eine sichere Unterscheidung dieser Fälle (Pseudorauschbrand, etc.) vom echten Rauschbrand ist auch für den geschulten Bakteriologen oft schwierig. Differentialdiagnostisch wichtig ist namentlich der Umstand, dass der echte Rauschbrand beim Rind gewöhnlich nur in den bekannten sog. Rauschbranddistrikten vorkommt und Thiere über 4 Jahre gewöhnlich nicht mehr befällt." On page 180 they say: "Die Unterscheidung stützt sich auf die beschränkte enzootische Verbreitung des Rauschbrandes gegenüber den allenthalben vorhandenen Oedembacillen, auf den gewöhnlich letalen Verlauf des Rauschbrandes und auf die characteristische Fadenbildung der Oedembacillen."

I think that these statements are traceable to Kitt (*Münch. Jahresberichte*, 1885, p. 39).

I conceive the broad epidemiologic factors in these diseases to be as follows: In the soil in general, wherever life processes occur to any appreciable extent, organisms of the anaerobic group are to be found. The vast majority of these organisms are probably not truly parasitic at all. Different species possess different powers of tissue penetration. To some extent different animals possess different defensive powers against the various species of anaerobes. In general the most highly pathogenic species of anaerobes are comparatively rare in their distribution and wounds do not become heavily contaminated by them and do not frequently become infected. But in regions where animals have died of anaerobic infection since time immemorial there occurs such heavy contamination of wounds by anaerobic organisms that such wounds frequently become infected, and animals ingest quantities of pathogenic organisms, and the chances for infection are multiplied, and whatever be the factors promoting infection, animals occasionally develop the disease, and the district becomes known as a "Rauschbranddistrikt."

Friedberger and Fröhner say (320): "Der Rauschbrand ist eine stationäre, dass heist an gewisse Oertlichkeiten gebundene Infectionskrankheit des Rindes, welche auf bestimmten Weiden und Alpen, so wie in gewissen Ställen das ganze Jahr hindurch, namentlich aber in den wärmeren Monaten, also in Juni, Juli, und August, zur Sommer und Herbstzeit herrscht und besonders auf sumpfigen Terrain vorkommt." This type of statement permeates the literature. It opens ground for much speculation.

That valleys and meadows and swamps should be the most dangerous places is easily understood, when one considers that they are the most closely grazed, that valleys are natural highroads, and that mud is a very active agent in the promotion of wound infections. Then, too, it must be remembered that the disease is one of summer time, in other words, of the grazing country. Doubtless farmers are more careful to destroy carcasses of animals dying near the stables.

To what extent excessive infection of the soil is due to other factors than the contamination of the soil by dead animals, probably no one is at present prepared to state. Le Clainche and Vallée showed that blackleg organisms were present in the feces of cattle which inhabited "blackleg districts." The carelessness of stockmen in the disposal of carcasses is certainly a factor in the spread of these diseases. District veterinary Kjoss-Hanssen of Egersund, Norway, states that as soon as sheep are known to have Bradsot the peasants slaughter them, the bodies are butchered, the intestines are thrown to the wind and weather or at most buried in mounds, wherefore contagion is spread over the fields as in the old days. Hamilton, (a, 292) speaking of the Scotch shepherds, says that if the body of a braxy sheep is so decomposed that it is unfit for food it is left in the fields after skinning, or may be thrown on the manure heap. Marsh speaks of a farmer in Wyoming who let 20 carcasses of sheep, dead of blackleg, lie long in a field. Wulff (673) says: "Bis vor wenigen Jahren wurde diese Krankheit (Geburtsrauschbrand) allgemein dem echten Rauschbrande zugezählt, und noch heute vertritt mancher Praktiker (Honecker) den Standpunkt, dass diejenigen Fälle, die nicht als puerperales malignes Oedem positiv erkannt werden, als Rauschbrandverdachtsfälle wie Rauschbrand veterinärpolizeilich zu behandeln und zu entschädigen sind." It appears to me that Wulff is entirely wrong in implying that there is no need of destroying bodies of animals dead of malignant edema infections. His point of view is directly traceable to the oft quoted idea that the bacillus of malignant edema is universal in its distribution and contaminates every open wound. This in turn is due to the contamination of cultures of nonproteolytic pathogens with the universal proteolytic forms. I should suggest that it were a greater crime against society to leave unburied or undestroyed a cadaver dead of a vibrion septique infection than one dead of a blackleg infection—the organisms of the vibrion septique group have a far greater range of possible hosts among domestic animals, and they are probably, in many cases, at least, quite as pathogenic for cattle as are blackleg organisms. Such laws as exist concerning the destruction of blackleg carcasses should most certainly be construed to cover carcasses of animals dead of both types of infection.

It is conceivable that one type of anaerobe may be responsible for most of the infections of a given district. Such a point should, however, never be taken for granted in any one case. It is also conceivable that one type—perhaps the blackleg type—may infect through the alimentary canal or by some unknown means with more facility than do the organisms of the other type.

It is evident from this discussion that the mode of infection in blackleg, beyond its being an occasional contamination of gross wounds, is entirely unknown. Most discussions as to the portal of entry of the blackleg bacillus revolve around wound infection of some sort, external or alimentary. Because of the fact that blackleg organisms do set up local processes where they are inoculated, and because in braxy the site of inoculation and the site of maximum proliferation is apparently in the alimentary canal, it seems highly improbable that blackleg is frequently a primary alimentary canal infection. It appears to me that the possibility of insect transmission of blackleg has been seriously neglected.

Insect Transmission of Blackleg and Allied Diseases: I have discovered no reference in any textbook to the rôle that may possibly be played by insects in blackleg infection. The idea of such a mode of infection is, however, an old one.

Hintermeyer describes an epidemic of quarter-evil, which raged in the summer and autumn of 1846 among the deer of the park of Duttstein, which Budd quotes somewhat inaccurately: "Als wesentlich habe ich hier noch zu bemer-

ken, dass die Bremsen, und zwar (a) die grosse Rind-bremse (Tabanus bovinus), (b) die Regen-bremse (Tabanus pluvialis), und (c) die Blind-fliege (Tabanus coecutiens) welche im verflossene Sommer in unzähliger Menge vorhanden waren, wohl mit Recht als die theilweisen Träger des Contagiums anzusehen sind, und daher einere grössere Ausdehnung der Seuche verursachten. Diese setzten sich gewöhnlich zu Tausenden, auf die Cadaver der gefallenen Thiere, saugten die aus Maul, Nase, und After kommenden Profluvien ein, verliessen sodann die Leichen, begaben sich sofort auf gesunde Stücke stachen ihren von Contagium Saugrüssel in die Oberfläche der Haut ein, und inoculierten auf solche Weise das Seuchengift." We cannot be sure, of course, of the identity of this epidemic.

Guillebeau suggests that the larvae of bot flies (Hypoderma bovis) may cause infection of cattle in their wanderings from the alimentary canal to the skeletal muscle and to the skin; they make minute canals through the flesh. I have found no confirmation of this suggestion in necropsy reports. Eggimann states that there is no Rauschbrand in Emmentahl and there are few warbles in hides from there, while skins of cattle from other valleys contain warbles.

I have found only one reference to experimental work in insect transmission of blackleg. Sauer caught common flies and biting flies which had passed a Rauschbrand cadaver. A small number of heads of these animals sufficed to produce typical Rauschbrand in guinea-pigs when inoculated subcutaneously. The bacilli were shown to multiply in the bodies of the flies, but the newer generation were smaller and weaker. Flies which had been on Rauschbrand cadavers were placed with wounded guinea-pigs, and they infected them in two cases. It was not shown whether the bites of flies could infect guinea-pigs.

A Laboratory Infection by an Organism of the Vibrion Septique Group.—I think the possibility of anaerobic infection by a small number of organisms entering through a minute wound, as would be the case in insect inoculation, has been overlooked. In this connection I may state that I recently infected my forefinger at the distal segment with a few washed organisms of a 24-hour culture of strain AS, of the vibrion septique group. The wound was a minute prick with a fine capillary pipet, and it was not more than 2 mm. deep. The finger became painful in 5 hours and swelled slightly, but was not feverish nor did it become cooler than the others. It was carefully kept warm to foster an active circulation. Twenty-four hours after the inoculation, however, on account of neglect, the finger became chilled and it immediately swelled perceptibly. Two hours later it was immersed in hot water and the swelling went down somewhat. Pain and swelling remained in the distal segment of the finger for a month, though fever and a tendency to suppuration were not noticeable. Apparently the infection lingered in the synovial membranes or in the tendon sheath. This is a case of invasion by an organism of the vibrion septique group in which no necrotic tissue was present, in which the size of the wound was infinitesimal, in which the depth of the wound was slight, in which no foreign débris was introduced, and in which no appreciable amount of culture toxin accompanied the organisms. But it will be noted that the organisms introduced were in the vegetative state and had been actively multiplying shortly before they were inoculated. I am confident that had circulation for some reason been partly suspended in the infected finger, the results would have been serious. I have found no other record of a laboratory infection by anaerobic invaders.

Perhaps it will not be considered mal apropos if I also introduce a few references as to transmission of anthrax by insects.

Virchow says: "Most commonly insects with piercing probosces effect the inoculation, such as gadflies (bremse); but flies which make no wound may also implant the poison on the skin by their soiled wings and feet."

Bourgeois expresses himself to the same effect.

Budd (1863) gives various points indicating insect transmission of anthrax. All but one of the cases of malignant pustule of the face which he has seen occurred in hot weather, in summer and autumn, while malignant pustule of the hands occurs all the year around. He cites two cases of women who were bitten by flies and developed malignant pustule at the site of the puncture, and another case which was caused by the puncture of a gadfly that came out of a fleece of wool.

Raimbert concludes (1869) from certain experiments that anthrax may be transmitted by flies, and that it is more probable that it is transmitted by flies that feed from cadavers than from flies that live by bloodsucking only.

Koch states that practically all the anthrax of the Russian steppes occurring at harvest time is due to insects, but does not give proof of his statement.

Minzmain (1914) successfully infected guinea-pigs with anthrax by means of the biting flies Stomoxys calcitrans and Tabanus striatus. The flies were applied to the hosts shortly before the death of the latter, and immediately after or 10 minutes later were applied to healthy guinea-pigs. The exposed animals died during the evening of the third day of typical anthrax.

Morris infected wounded guinea-pigs with anthrax by means of nonbiting flies.

As far as I can make out there is a generally accepted opinion among entomologists and veterinary scientists that anthrax may be, and frequently is, transmitted by flies.

The nature of blackleg is not so different from that of anthrax. The anaerobic organisms in the vegetative form are easily killed by air, as I have found in using the Barber technic of isolation. It is possible that inoculation of spores even in small numbers may prove infectious, but it is my opinion that inoculation of vegetative rods is much more so. If the statements of Sauer are correct, we may conclude that vegetative forms of the bacillus may be found in the bodies of flies. It is my opinion that insect transmission of blackleg is exceedingly likely. When one sees the charts representing the incidence of blackleg in "Le charbon symptomatique du boeuf"; how the malady increases in May and reaches its zenith in July, decreasing and practically disappearing in some years in September and in others in October, one is strongly inclined to look no further for a means of transmission of the disease. But it must be borne in mind that summer is the time of abundant grass in Switzerland. In California, Dr. Traum assures me, blackleg is to be expected whenever the grass is abundant, in fall or spring as the case may be. In Switzerland (Arloing, Cornevin, and Thomas) anthrax and blackleg occur at the same time; in California anthrax occurs in summer and fall when the ground is bare. It has frequently been noted that the fattest cattle die of blackleg, the finest sheep die of braxy. The explanation has been sought in the greater abundance of sugar in the muscles of animals that are well conditioned. Möller states that guinea-pigs that are sugar fed show much gas on blackleg inoculation, those that are not sugar fed show less, those that are starved show none at all. Guinea-pigs that are starved are less likely to succumb to blackleg than those that are well fed. I should say that this element must be thought of as a predisposing factor and not as a factor leading to infection.

Farmers in some portions of California often attribute blackleg infection to the drinking of contaminated water. Farmers of other districts in which the water is excellent are emphatic in asserting that the infection is in the food. There is not necessarily a single avenue of infection in these diseases, no more than there is in anthrax, and the demonstration of one mode of infection does not prove that it is the only or usual mode.

One strong point opposing the view that vibrion-septique infections are not rare in cattle is the apparently comparatively satisfactory protection furnished cattle by vaccination. Kitt (a) states that in Bavaria between the years 1898 and 1907, 0.144-0.320% of vaccinated cattle died of blackleg and 0.551-2.497% of unvaccinated cattle died of blackleg. Balavoine's figures for Switzerland are not very different from Kitt's. Blackleg aggressin is said to protect cattle even better than the bacterial vaccines. To what extent vibrion septique strains have been unintentionally used for vaccination purposes it is at present impossible to estimate.

The epidemiology of these infections is one of the most interesting in human and animal pathology. I regard it as an almost virgin field for investigation. It should be studied, and studied exhaustively, not by the veterinary pathologist, but by the trained anaerobist, who will investigate thoroughly every case that comes under his observation, and will not be satisfied that the first organism that comes to hand is the only one involved in the death of the animal before him, until he has looked carefully for others. Under the investigation of the epidemiology of blackleg and malignant edema should come the investigation of the immunity of older animals to these diseases, the immunity of animals from districts free of blackleg and braxy, the relative immunity of cattle and sheep of different breeds, infection of wounds and infection through the alimentary canal, infection by means of insects, and infection through causes that are now unknown to us—innumerable problems could be suggested. We shall not, however, be in a position to make such problems a profitable study until more is known as to the specific members of the groups involved.

(c) Conclusions as to the Anaerobic Infections of Cattle.—The conclusions as to the anaerobic infections of cattle which I derive from study of the above mentioned material and from a study of the literature of the subject of animal infections are:

Theoretical considerations lead one to conclude that cattle infection by a single species of anaerobe may take place, and that this species may be an organism of the vibrion septique group, of the blackleg group, or of the oedematiens group; or a mixed infection may take place by members of the different groups or by two members of the same group, or some other combination of organisms may invade the tissue. I find no record of B. Welchii infection in cattle. There is probably no reason why it may not be found as a wound infection, nor why other organisms may not be found, nor why proteolytic organisms may not also occasionally invade bovine tissue with the non-proteolytic invaders as they are known to do in man and small animals.

Probably some of the ideas concerning the proteolytic nature of malignant edema are due to the observation of such cases.

Practical considerations lead to the following conclusions: Cattle are frequently subject to spontaneous infection by members of two groups of anaerobic invaders, the blackleg group and the vibrion-septique group. Both types of these infections are, in the vast majority of cases, diagnosed as "blackleg," "Rauschbrand," or "symptomatic anthrax." Infection by members of the first group is commoner than that by members of the second group, but infection by members of the second group is by no means to be ignored and probably should be considered in cattle immunization.

It is possible that a member of one group may be the predominant invader in one district, and a member of the other in another, which indicates the necessity of determining the bacterial agents involved in "blackleg" infection in various districts.

It is also possible and, to my mind, probable, that factors exist that induce anaerobic infection in cattle, whose identity it is important to solve. Beyond the knowledge that these infections may follow wound contamination we know nothing positive as to the mechanism of anaerobic infection in cattle.

Careful, intelligent, and thorough bacteriologic investigation of fresh cadavers of many animals dying of spontaneous "blackleg" infection is much needed, and connected with it is the need of an exhaustive epidemiologic survey of the field. The attitude of the veterinary profession has been that we already possess all the necessary knowledge concerning blackleg. It appears to me that we are in the morning of the investigation of blackleg and allied diseases, and that what we today know is but an inkling of the true state of affairs. An investigation of the subject that will properly correlate and solve the problems involved can be undertaken only after many workers have made studies of many cases from different districts. The reasons why this has not already been done are two: anaerobic technic has not been adequate for such study, and the idea of the monospecificity of these invasions has tenaciously held the field.

(f) The Immunization of Cattle.—It is not my purpose here to enter into a discussion of the extensive subject of the immunization of cattle against blackleg by bacterial products.

Arloing, Cornevin, and Thomas were the pioneers in immunization by killed and attenuated virus. Kitt modified their technic and also introduced serum virus inoculation for the production of immediate protection. His article in Kolle and Wassermann's Handbuch (IV, 1913) may be referred to as a modern authority on the subject. The use of filtrates for immunization of cattle was first suggested by Roux in 1888 (b) on the basis of protection afforded to laboratory animals by filtrates of infected guinea-pig tissue. Today various means of immunization are in use. Injection of powdered attenuated muscle virus and of pellet vaccines is still widely practiced, and tissue and culture filtrates, the so-called "aggressins," are apparently gaining in favor because of the safety attendant on their inoculation. Serum immunization is used for herds in which blackleg has recently occurred.

Suffice it to say that immunization of cattle against blackleg is regarded as highly necessary in some districts and as advisable in others. But it must be emphasized that such immunization is not always successful and that inoculation infection ("Impfrauschbrand") is not uncommon. These facts fit in so plausibly with my own figures and with the thesis of this paper that I am forced to conclude that the subject of the immunization of cattle deserves a careful investigation.

Franklin and Haslam state: "Complaints had been received concerning all vaccines in common use. Part of these involved losses of calves immediately after vaccination, others death from blackleg in spite of numerous vaccinations" (J. Infect. Dis. 1916, 19, p. 408).

The only case of inoculation blackleg that has come to my attention is that of Dr. Haslam (private communication). The causative organism was a member of the vibrion septique group of the general type of the strain called AS. The spores sometimes lie sidewise in the bacillus and this type of organism is one of the most highly invasive and highly pathogenic of the group, and grows unusually actively on meat medium. The calf from which this strain was derived was one of several killed by a pellet vaccine. It is conceivable that such an organism as this would be insufficiently attenuated by the conventional procedure, which satisfactorily attenuates organisms of the blackleg group. Careful observation of details of this sort will perhaps clear up most cases of inoculation blackleg.

Animals immunized against the vibrion septique group are not immunized against blackleg, and vice versa. All immune reactions separate the two groups. So far as I am able to discover by a careful search of the literature, there is no sign of failure on the part of one strain of a group to immunize against other strains of the same group.

Robertson (b) found that the four types of vibrion septique which she distinguished on the basis of the agglutination reaction, all produced a toxin which was neutralized by a monovalent antitoxin.

It is my present opinion that cattle should be immunized against both groups, but perhaps such practice should depend on the nature of the organisms found to infest a particular locality. Vaccine for the vibrion septique type should be made separately from blackleg vaccine and probably should be more severely attenuated. It must be emphasized that the worker in charge of the manufacturing of such vaccine should be thoroughly familiar with both types of organisms and should check his cultures continually.

2. THE ANAEROBIC INFECTIONS OF SHEEP

Sheep infections may be considered under four heads: (1) blackleg; (2) malignant edema or vibrion septique infections; (3) braxy or Bradsot, and (4) black disease.

(*a*) *Introduction.*—Distinction between these infections is extremely difficult, if we are to judge from the accounts in the literature. One is inclined to make the statement that the nature of the diagnosis depends more on who describes the disease than on what it is. One point to be brought out is that clinical characters have been so emphasized in connection with the different sheep infections that etiologic factors have been largely lost sight of. Another point for consideration is that gangrenous infections of sheep are perhaps different from gangrenous infections of cattle; that is, the specific nature of the host must be considered in diagnosis, as well as the specific nature of the parasite. A possible difference between infections of cattle and infections of sheep appears to be the greater selective preference of the organisms for mucosa of the fourth stomach and the intestines of the sheep. It may well be, however, that closer study will show that this difference is one referable solely to the specific invaders or to the position and nature of the portal of entry of the infection.

Another difference that may exist between sheep and other animals has probably been the cause of much confusion as to sheep infections. This is the phenomenon of the rapid decomposition of sheep cadavers. While cadavers of cattle dead of spontaneous anaerobic infection and those of experimental animals dead of nonproteolytic anaerobic infection show a marked slowness in decomposition, those of sheep are so frequently referred to as readily decomposing that one must conclude that the nature of the sheep in this regard is different from that of other animals. Krabbe, in 1875, mentions the fact that sheep dead of Bradsot decay so rapidly that dogs and ravens will not eat them. On the other hand, we frequentlly hear that the flesh of cattle dead of blackleg may be eaten by man. Jensen, Hamilton, and Gilruth all mention the rapid decomposition of cadavers of sheep dead of braxy. Gilruth (b, 572) goes into some detail in the matter.

He says: "Considering the bacterial flora present in the large intestine of all animals, not excluding the sheep, it would be surprising if even prior to death in such a disease other putrefactive bacteria did not reach the peritoneal effusions. That sheep, especially fat sheep in wool, rapidly putrefy after death, from whatever cause, is common knowledge to all sheep men. I have seen carcasses of sheep dead of suffocation in trucks putrid and emphysematous, the skin being livid, in six to ten hours. Even in cold weather I have detected putrefactive organisms in fair numbers in the blood of the right heart four hours after death by accident, and these must have traveled from the intestine by way of the blood stream. The wool, in addition to the subcutaneous fat, makes of the animal a perfect incubator for putrefactive organisms by preventing dissipation of the body heat." This account strongly suggests that the blood and lymph of normal sheep, or at least that of sheep about to die, contains bacteria. The blood of horses is known to contain bacteria after eating. If this difference between the flora of sheep and that of other animals exists, it accounts for the fact that from anaerobic infections of cattle and hogs nonputrefactive organisms are usually easily isolated and that in the case of sheep infections we have few clear statements of the cultural characters of the organisms isolated, while many indications point to the fact that mixed cultures have been considered the causative agent of the disease. The fact that sheep infections are usually alimentary canal infections, while cattle infections usually are muscle invasions, would also play a part in causing this difference. Such reports as I have seen, wherein several species of bacteria were observed in the tissues of sheep dead of black disease or of braxy, made no mention of recognition of the polymorphism of certain species of anaerobes, so that one finds himself suspecting the opposite error to the one usually to be considered, namely, that a worker may have grouped several species as one. I have the word of an experienced meat inspector that slaughtered mutton keeps better than pork but does not keep so well as beef. Anaerobic hog infections, however, are apparently easily referable to monospecific invasion. Gilruth believes rapid decomposition to be due to the nonconductivity of the wool and fat, and, until further information comes to light, this must be considered as probably the sole cause of rapid decomposition of animals dying by accident. It must be remembered, however, that such a cause does not eliminate the probability of occasional collateral invasion of sheep tissue by proteolytic anaerobes with the nonproteolytic ones; this type of invasion occurs frequently in wound infections and in artificial inoculation of guinea-pigs, and, so far as can at present be judged from the literature, it occurs frequently in sheep.

(*b*) *Blackleg in Sheep*.—It is evident that many diagnoses have been made of blackleg in sheep when the diagnostician did not know the difference between blackleg infections and malignant edema or vibrion septique infections in sheep. This is, of course, natural, as many diagnosticians do not know the difference between blackleg in cattle and malignant edema or vibrion-septique infections in cattle. Textbooks frequently mention blackleg in sheep, but the actual notes of the occurrence of this disease are rare.

Regn mentions five cases of experimental Rauschbrand in sheep, which cases he evidently did not himself see. He worked with material taken from them. One gave an organism that he admits was proteolytic and nonpathogenic for guinea-pigs, but which he insists was Rauschbrand. The other samples contained "typical Rauschbrand." His criteria for the diagnosis of Rauschbrand are so seriously open to criticism that it is impossible to be sure what the sheep really died of. Rauschbrand, according to Regn, produces large spherical woolly colonies in various mediums in a short space of time, though occasionally his strains produced small lenticular colonies which were proved to be Rauschbrand also. According to Regn, Rauschbrand produces in the guinea-pig gas and injection of the intestines and of the peritoneum, and forms chains on the liver of that animal. Regn proves that his organism is not the malignant edema of Koch because it does not cause the production of a proteolytic odor, and quotes von Hibler to that effect, not noticing that von Hibler describes also the bacillus of Ghon and Sachs, which Regn's organisms resemble much more closely than they do Rauschbrand, and in spite of the fact that von Hibler expressly states in all his publications that Rauschbrand does not produce woolly colonies or filaments on the liver of animals.

Scheibel says that blackleg is often observed in Upper Hesse in sheep and goats. Hutyra and Marek (42) state that blackleg is pathogenic for goats and sheep.

Moore discusses blackleg of sheep. "Sheep are said to be more susceptible to experimental inoculation of blackleg bacteria than cattle, but outbreaks of the disease among them are not common. It is believed, however, that it may be overlooked or mistaken for other affections. Stockman (no reference) found the lesions of the skeletal muscles to be very slight, or even absent, in sheep dead of blackleg." The symptoms, according to Scheibel, are given, but no reference is made to blackening of the muscles.

Kitt (c, 296) says: ". . . ich habe wiederholt grosze mengen virulenten frischen Fleischsaft, Fleischbrei, getrocknetes Fleisch von Rauschbrandigen Rindern vergeblich jungen Rindern und Schafen verfüttert. Merkwürdigerweise krepierten einmal kurz hinter einander mehrere Schafe, die in dem ungereinigten versuchstall auf dieselbe Matratzenstreu eingestellt wurden, auf welcher vorher rauschbrandgeimpfte Schafe verendet waren, an typischen Rauschbrand, und zwar zeigten sie auszer characteristischem Bazillenbefund des Fleisches auch hämorrhagische Gastroenteritis. Vielleicht ist der Grad der Sporenreife oder die jeweilige toxische Virulenz des Erregers maszgebend für die intestinale Infektion."

It must be suggested that true blackleg does not produce marked gastroenteritis in the guinea-pig, nor did it in the sheep on which Gunn performed necropsy examination (see below), though it may do so on intravenous injection (Arloing, Cornevin, and Thomas). Kitt does not mention blackening of the muscles of the sheep. On page 297 he says: ". . . in der Bauchhöhle wachsen anscheinend auch die Rauschbrandbacillen beim Meerschweinchen zu längeren Fadenverbänden aus und ist die Unterscheidung von Pseudorauschbrand schwer." I have, as stated above (p. 4), not found a strain of blackleg that formed filaments on the liver of the guinea-pig, neither does von Hibler report them. Kitt also says (300) that in 12-36 hours in the incubator Rauschbrand colonies are visible, and that they form enough gas to force the plug out of the tube. I have not found that true blackleg strains grow in any such fashion. I would also suggest that of Kitt's three photomicrographs of Rauschbrand bacilli in "Bakterienkunde" only one, "Klostridiumformen in Blutboullion," resembles my strains morphologically. The third photograph shows an organism with spherical spores, as well as one with oval spores. I have not seen spherical spores in a pure culture of blackleg or of vibrion septique of any type. Whether Kitt's sheep infections might not have been termed "Bradsot" by a pathologist from a Bradsot district would be of interest to know.

Hadleigh Marsh reports an outbreak of blackleg in sheep. His case is that of a flock of rams into which a ewe was introduced by mistake. The rams fell to fighting and many of them died of a disease which he identified as blackleg.

Necropsy examination on one of the animals was made by Dr. Gunn, who reported the following findings in a letter to Marsh.

"A distinct crepitant swelling on the left rump. Subcutaneous lesions dark red and infiltrated with gelatinous exudate similar to blackleg in cattle. On incision the muscles showed the typical picture of blackleg and gave off gas bubbles with the typical smell. Precrural lymph glands as well as popliteal, iliac, and ischial glands were acutely swollen and filled with the same edematous fluid. No marked changes in thoracic cavity or abdominal cavity. Lungs, liver, spleen, kidneys, and intestines practically normal." Marsh kindly furnished us with material from this sheep. The invasive organism isolated was typical blackleg (strain RM). Others of the twenty sheep were not studied.

Witt and Stickdorn report careful determination on sheep dead of anaerobic infection. They diagnose the infection as Rauschbrand and apparently have adequate reasons for so doing. They raise the question, Is sheep Rauschbrand identical with cattle Rauschbrand? Witt defines the characters necessary for the diagnosis of Rauschbrand in sheep. These include the characteristic blackleg odor, sponginess of the muscle and black or reddish-black coloration of the muscle. Gastro-enteritis is not mentioned in the review quoted.

Strain RG, which I isolated from a sheep thought to have died of Bradsot on the estate of Graf Bülow in Halle (a sample of the muscle of which animal was sent me by Dr. Jensen), is a blackleg group strain. It is a species distinct from any other in my possession and fulfils all requirements as to blackleg characters as stated on page 4, a.

Various authors report experimental infection of goats with blackleg, but I know of no accurate report of natural infection of goats with this disease.

(*c*) *Malignant Edema and Vibrion Septique Infections of Sheep.*—Records of these infections show that there is a group which must be classified as wound infections.

Haubner describes a disease that he calls "Feuer der Schafe" and which Friedberger and Fröhner believe to be malignant edema. Haubner thinks that the disease is due to wounds of the legs caused by the sheep dogs, and the disease also commences at the site of the bite and the accompanying hemorrhage. It occurs especially in fall when dry fodder is first fed. It appears suddenly as a painful edematous swelling of the skin and connective tissue of the inner surface of the hind leg, more rarely of the front leg, neck and submaxillary region; the affected animals limp and have systemic feverish symptoms. The swelling spreads rapidly from the thigh to the belly and breast, the skin becomes bluish red, gas formation takes place, the animals die in 18-24 hours and at necropsy show bloody infiltration of the subcutaneous connective tissue, with gas, hemorrhages in various organs, slight putrefaction, dirty brownish blood which does not clot, and no spleen swelling.

Lembeken saw 50 of 600 freshly shorn sheep die of malignant edema.

Hutyra and Marek (34) class sheep and goats as less susceptible to malignant edema than horses.

J. A. Gilruth, then chief veterinary of New Zealand, described in 1893 "Blood Poisoning (Malignant oedema) in Sheep and Lambs in New Zealand." It is distinctly represented as a wound infection which occurs after shearing, docking, and castrating. In his report of 1900 Gilruth demonstrated the cause of the disease to be Vibrion septique Pasteur.

Foth (b, 212) describes an infection of a sheep that apparently died of blackleg. The anaerobic invader was, however, a chain forming organism.

(*d*) *Braxy or Bradsot.*—The best account of this disease that I have found is that given in the Reports of the Journal of Comparative Pathology and Therapeutics (1902, 15, p. 181). I presume that this review is to be attributed to one of the editors of the journal, Dr. J. M'Fadyean or Dr. Stockman. I quote rather liberally from this paper because some of the sources are inaccessible to me. It is primarily a review of a paper by Professor Hamilton of Aberdeen University, to whom the reviewer refers as the "author." Hamilton finally identifies the braxy of Scotland with the Bradsot of Norway, Denmark, Iceland and Germany. There have been many papers on the disease which lack satisfactory evidence as to its nature (182). "The first really scientific account of the pathology of the disease is that by the Norwegian Government veterinary officer, Ivar Nielsen, who likewise must be regarded as having discovered the micro-organism which is its cause. Previous to the publication of Nielsen's paper in 1888, and even for some time afterwards, the malady was considered to be anthrax."

". . . braxy is certainly not anthrax. . . . There is another disease, however, with which it has even closer points of resemblance, namely, quarter evil (Rauschbrand). Quarter evil is said to be unknown also in Iceland, and the two diseases, and the organisms causing them, when compared, are readily enough distinguishable. Thus braxy is never accompanied by any external lesion, as is the case in quarter evil; it also runs a much more rapid course than that disease.

"Braxy is, therefore, neither an anthracoid disease nor is it to be confounded with any other malady; it is a disease *sui generis*."

(p. 183) "Nielsen has defined it as a gastromycosis, excited by a specific bacillus introduced with the food into the fourth or true-stomach, and believes that the disease may either pass into a general affection, or may occasion the death of the animal through absorption of bacterial toxines formed by the organism germinating locally in a part. In these views he was supported by Jensen, who concluded as a result of the researches of previous investigators that bradsot is an acute or even extremely acute, infectious disease which begins as a hemorrhagic inflammation of the mucosa of the fourth stomach, which is accompanied by the formation of gas in the alimentary canal, especially in the stomach, and which kills the animal in some cases by a general infection, in others apparently by toxic poisoning, or possibly in others still by dyspnea caused by tympanites.

"He held that the bacillus is introduced with the food, and that braxy thus differs materially from quarter evil, which, according to Kitt's experience, he says, is seldom or never induced by administration of the organism by the mouth. He would also stigmatize the fourth stomach as the organ primarily and most severely affected. Too much emphasis has been placed on the stomach lesion as an essential feature of the malady. In Professor Hamilton's experience the sloughy hemorrhagic patches found on the mucous membrane of the fourth somach in some cases are completely absent in others, and the large inflammatory patches described as occurring on the outside of the rumen or other division of the stomach have nothing to do with inflammation, nor are they hemorrhagic. They are simply part of the general blood staining which ensues after death. No one, moreover, as Jensen himself confesses (see p. 67), has conferred braxy on the sheep or any other animal by feeding with the bacillus, and this result coincides with the author's own experiments. He has fed sheep on the blood, peritoneal liquid, and other liquids taken from animals dead of braxy, and which were teeming with the bacillus, and has never once succeeded in conveying the disease to the fresh host by this means, while the same liquids injected subcutaneously killed the animal in a few hours. Jensen gets over this difficulty by affirming that the herbage at the time braxy breaks out is cold, often of a rough nature, and a good part of it indigestible, and that this predisposes the animal to infection from the stomach.

"The author thinks, however, that too much importance has also been attached to the lesion sometimes found in the stomach as evidence of the disease being communicated through that organ, for he has found in animals which died by the introduction of the bacillus experimentally under the skin of the thigh, that sometimes the sloughs on the stomach were most typical. His impression is that it is a general disease, and that very soon after it is established the whole of the liquids, organs and tissues become more or less beset with the bacillus. He has never failed to find it, and in greater abundance than elsewhere, in the serous and blood-stained effusion into the peritoneum, and in that contained in the pleural cavities."

(p. 184) "*Age of Animals Attacked.*—Accounts of the disease from all countries seem to uphold the allegation that the first year's animals are far more liable to braxy than those more mature."

(p. 185) "Sporadic cases are said to occur during the summer months, but the great range of mortality prevails during the late autumn and early winter months. It may commence in September, or be delayed till the first or second week of November—a good deal depending apparently on the advent of frosty weather. It disappears with quite as great irregularity—sometimes by the middle of December, at other times not until the end of February. The same peculiarity of advent and cessation of the disease has been noticed in all countries where braxy prevails.

Postmortem Appearance.—"According to Jensen's description, the following are the chief postmortem appearances:

When the animal is killed during an attack the essential change is a dark bluish-red, somewhat swollen patch on the fourth stomach. This patch increases in size, so that toward the end of the attack a great part or the whole of the stomach may be hemorrhagic or serous hemorrhagic. The fourth stomach and adjacent part of the bowel are devoid of food, but sometimes contain a bloody fluid. The hemorrhagic infiltration may spread from the fourth over the other stomachs, partly over the duodenum, or even over a great portion of the intestine, while other parts of the intestine may be hemorrhagically injected. In the cavities of the body there may be a little serous fluid. The blood is dark, and may be clotted; and the spleen is occasionally somewhat swollen, but may be quite normal. The liver is pale, brittle, and degenerated, and in extreme cases the friability is probably from *postmortem* causes. The kidneys may be normal or somewhat degenerated; not uncommonly, however, they are enlarged and very brittle or even diffluent. The cadaver decomposes soon, and before long the hind quarters become blown up with gas, the verge of the anus protuberant, the skin takes on here and there a bluish color, and the wool is easily detached; sometimes the skin bursts and a serous hemorrhagic fluid is seen oozing from the subcutaneous areolar tissue.

"In the case of sheep inoculated with a culture of braxy bacillus Professor Hamilton found that all showed a very marked hemorrhagic subcutaneous edema which spread out from the point of inoculation on the inner side of the thigh over the hindquarters and belly. In the musculature of the thigh and leg, partly in the abdominal muscles, there was widespread hemorrhage, to such an extent that sometimes the muscles were quite black. Emphysema was also very evident in the muscles. Within the abdomen there was a little blood-stained liquid. The spleen, in all cases, was slightly enlarged, the liver yellow and degenerated, and the lungs very edematous. In one lamb the kidneys were soft and degenerated while in other two they were almost diffluent, an appearance which he says is characteristic of spontaneous cases. On microscopic examination, great numbers of rods with round ends, and which were sporing, were found in the muscle juices."

(p. 186) "The surface of the mucosa is necrotic, and most of the sloughy half-detached material evidently becomes digested off. The epithelium was always found to be destroyed by auto-digestion, while the necrosis of the surface extended into the glandular structure of the mucosa for perhaps a fourth of the thickness of the entire membrane. The blood corpuscles in the minute hemorrhages were always in a state of dissolution, their presence being indicated by brownish pigment."

The lesions in typical cases are summarized by Hamilton:

"1. The absence of any external manifestation of the disease as in the case of the slough of quarter evil.

2. The tendency which there is, both during life and after death, to the production of gas.

3. The presence of sero-sanguinolent effusions into the various cavities of the body and into the subcutaneous areolar tissue.

4. The tendency to blood-staining of the tissues.

5. The absence of inflammatory manifestations.

6. The occasional, but by no means invariable, hemorrhagic infiltration of the mucous membrane of the fourth stomach, with, from time to time, ulceration or digestion of the surface of the infiltrated parts.

7. The distention with food of the first three stomachs, and the absence of food in the fourth or true stomach, and usually in the intestine. The presence of a little brownish-red grumous liquid in the fourth stomach.

8. The occurrence, occasionally, of hyperemia of the large vessels in the walls of this organ, accompanied by blood-staining of the surrounding tissues.

9. The absence of extensive hemorrhage into the musculature of the body; the absence of gas-production in the muscles.

10. The invariable presence of the braxy bacillus in the liquids, tissues, and organs of the body."

(p 187) "*The Organism.*—The merit of having thrown the first light upon the etiology of the disease is due to Ivar Nielsen. In his work on the subject, published in the year 1888, he described an organism which he found in the hemorrhagic areas of the digestive tract, as well as in the capillaries of the various organs, which is, without doubt, the essential factor in the disease. The organism in question is a rod from *2* to 6 mikrons long or longer, and about 1 mikron in breadth, with rounded ends. It is sometimes found, in cultures at least, elongated into a thread, but, in the tissues it usually takes the rod form. It retains the stain by Gram's method, and more readily when quite freshly removed from the body; it is perfectly immobile, both when taken immediately from the various serous effusions or the blood and when in culture. As it occurs in the various liquids of the animal, it has a great tendency to spore, often nearly every rod being in this condition. The spore is usually placed at one end of the rod, and when developing gives to this end merely an appearance of being thicker than the other, while, with careful focusing, a clear, minute, and highly refractile point may be detected in the midst of the tuberosity. As the spore enlarges it becomes more and more refractile, and takes on an elongated oval shape, and in this stage the organism has a characteristic drumstick appearance very like that of tetanus. Sometimes the spore is located in the middle of the rod, and occasionally a couple of spores of large size are seen in a single bacillus, one at each end. The individual bacilli may be either united at an angle when in juxtaposition, or their axes may lie parallel and in line, in which case they may resemble a single bacillus of unusually great length. Exceptionally they may hang in rows, but this is not common. In certain instances of the disease the spores are present only in small number, or may even be absent, and in such cases the virus appears to confer a mild attack. At any rate, Professor Hamilton has often found difficulty in inoculating the disease and producing a fatal issue when the organism is in this non-sporing condition.

"It seems to be a strict anaerobe, at least when first removed from its natural habitat."

"The organism of braxy is apparently very closely related to that of quarter evil and of malignant edema. The three form a group which stand isolated, and which have intimate mutual relationships. In fact, when growing in solid media there is much difficulty in distinguishing them.

"Its complete immobility seems to be one of the best features by which the braxy bacillus can be distinguished from the other two. According to Jensen, the braxy bacillus is also to be distinguished from that of quarter-evil by the fact that it is pathogenic to mice, pigeons, fowls, and pigs, animals which are insusceptible to quarter-evil. It is a remarkable fact, however, that these animals never take the disease naturally nor has the disease ever been known to affect the cow, the dog, or man. The sheep seems to be the only animal which is naturally subject to it."

Some interesting epidemiologic studies on the immunity of sheep in certain districts follow the above abstracts.

Fröhner described a bradsot-like disease in sheep. He observed the symptoms closely: the animals became depressed, had difficulty in breathing, their abdomens swelled, they were in terrible pain, and they ground their teeth and were restless. They died in a few hours. Decay appeared soon after death. The subcutaneous connective tissue of the head and neck and of the side of the rump showed a gelatinous red infiltration, the muscles of the neck and the back and the cutaneous muscles of the belly were those most affected. The mucous membrane of the nasal cavity and of the turbinates was deep red and swollen, the lymph glands were swollen and infiltrated, the heart muscles grayish red with brownish red spots, the pericardium contained much red watery fluid, the pleura of the ribs were a diffuse deep red and there were also spleen, kidney, and liver lesions. It is possible that these infections took place by way of the nasopharyngeal mucosa, as did the hog cases described by Meyer, as lesions in the head region are not mentioned in necropsies of animals dying of the form of the disease which affects the abomasum, and it is also possible that infection was caused by the bites of flies that could reach only the head of the sheep.

Gilruth (b and c) identifies certain sheep diseases of Victoria and Tasmania and New Zealand with the braxy of Scotland and the Bradsot of Scandinavia, and reports an investigation of the diseases of Tasmania and of Victoria. He makes, however, the mistake of presupposing that the etiologic bacterial agent of a given outbreak is the same in all cases, and therefore he studied carefully only one strain from Tasmania (from a young sheep found dead and on which necropsy examination was performed before it was entirely cold) and one strain from Victoria (from a two year old wether, body almost cold, weather cold, rigor mortis present). As the records of braxy-like outbreaks, together with the fact that it is almost impossible to produce braxy by feeding, show that there is some mechanical or physical factor involved in the production of the infection, it follows that the infecting organisms in different animals dying of such diseases as braxy cannot be considered identical, unless they are isolated, studied, and proved to be identical. This postulate is held with truly infectious diseases; it is far more to be emphasized in the case of anaerobic diseases. Gilruth isolated from a Victorian case and from a Tasmanian case highly pathogenic anaerobic nonproteolytic strains which behave similarly but not identically: They immunize animals against each other and apparently do not immunize against blackleg, and animals are not immunized against braxy disease by malignant edema. These latter experiments are not extensive enough to be convincing, especially as the identity of the blackleg strain used must be called into question because it produced in sheep intense congestion of the abomasum and the intestines and formed woolly colonies; and as the purity and identity of the malignant edema strain must be called into question because, on inoculation of a culture (dose?), it produced a lesion in a sheep, from which the animal recovered. It is quite evident that the Tasmanian sheep died of a mixed proteolytic and nonproteolytic infection, but the Victorian sheep from which the bacillus was isolated probably did not. A Tasmanian sheep which was killed when very sick showed pronounced bacterial invasion with a primary focus in the abomasum, which was attributed to a wound caused by a nematode.

I am entirely at a loss to place systematically the organisms described by Gilruth. On the whole, his work, as far as it goes, is careful; his protocols are minute and show close observation. The weakness of his investigation lies in his unfamiliarity with other species of anaerobes and with the habits of anaerobes. His organisms do not resemble any with which I am familiar, but the descriptions which he gives are not extensive. Moreover, there must always be a strong suspicion of impurity of culture when an anaerobe strain diminishes in pathogenicity as do Gilruth's, even though the culture may not smell proteolytic. The only photomicrograph given us shows organisms more nearly referable to the vibrion septique group than to any other, and Gilruth continually refers to long filamentous organisms, which he takes to be contaminations. It would also be remarkable that more gas formation were not mentioned by Gilruth if the organism belonged to the vibrion septique group. The amount of clear subcutaneous edema produced by his organisms is also unusual for the vibrion septique type of organism. That the bacillus may belong to the oedematiens group, or that Gilruth's infections may have been mixed ones, are possibilities which must be considered.

Gilruth makes some interesting epidemiologic observations. He connects outbreaks of braxy with dryness and bareness of the soil and with forced ingestion of soil. In common with others he notes that the animals affected are young and in excellent condition. He produces a relative immunity to inoculation by feeding culture to sheep, and holds that this fact disproves, in the present state of our knowledge, the possibility of natural infection by the digestive tract. I cannot agree with this latter statement. Gilruth failed to produce braxy by feeding cultures in a gelatin bolus which contained glass fragments.

Miessner (quoted from Jensen, below) published a paper on the etiology of Bradsot in Germany. According to him the lesions produced by Bradsot in Germany are not as uniform as in the North, the mucosa of the fourth stomach are less inflamed or not inflamed at all, and bacilli are not always present. His conclusions were that the so-called bradsot bacillus was not the cause of the disease and that the etiology of Bradsot was entirely unknown.

Titze and Weichel report extensive experiments with Bradsot. Their history of the subject is detailed. They agree with their predecessors that the Scandinavian Bradsot and the Scotch braxy and louping-ill closely correspond, but state that no common etiologic factor has been demonstrated for them. (Louping ill was probably wrongly associated with braxy by Hamilton.)

They then discuss all reports concerning Bradsot in Germany and come to the conclusion that in Germany the disease presents the same characteristics as in the other countries, including the predominance of the disease in winter, but that most of the German cases occur when the sheep are shut up instead of when they are put out to pasture. Moreover, animals of all ages sicken in Germany. The occurrence of the disease is sporadic.

Titze and Weichel report observations on the cadavers of eleven sheep that died of a disease diagnosed as Bradsot. These cadavers were partially putrified, they showed the general pathologic characters of Bradsot; three showed, however, no changes in the mucosa of the fourth stomach; changes in the intestinal walls are not mentioned; bacteria were present in all the organs and in the blood; they resembled bradsot bacilli morphologically and culturally, but it must be remembered that the cadavers were not fresh. Organs from these sheep killed sheep on subcutaneous inoculation with a local reaction like that of "Impfrauschbrand"; the organs were fed to several sheep, which were not affected thereby.

Titze and Weichel then investigated several strains of "Bradsotbacilli" that had been isolated by others. They make the observation that these strains are not all alike. Their bacteriologic observations are not, however, very extensive.

Perhaps another word as to the anaerobe cultures, which are usually sent from laboratory to laboratory, would not here be amiss. Most anaerobe strains, if they are isolated in pure culture, become contaminated after a few transplants, if they are handled by workers who are unfamiliar with anaerobic methods and who do not recognize contamination when it occurs. Many cultures of current strains of anaerobes were probably never purified in the beginning. All reports on cultural work with anaerobes are practically valueless unless the worker is able to demonstrate that the technic of isolation used by him was an adequate one; that he is acquainted with careful anaerobic methods; that the strains behave consistently in his hands. It would probably be safe to say that 95% of the anaerobic cultures in circulation up to the period of the war contained other organisms besides the one they claimed to contain, and that 30% of them no longer contain the original species for which they were named. I do not make these charges lightly or rashly, but base them on a study of a vast amount of material.

The variations observed by Titze and Weichel were such as any one would find in a group of anaerobe cultures of the same name taken from various sources.

Titze and Weichel undertook extensive feeding experiments with sheep. Their methods seem reasonable, they fed cultures of bradsot bacilli and organs of sheep dead of Bradsot, always with negative results, trying them on hungry sheep and on fed sheep, exposing some of the animals to cold after feeding. Apparently, none were shorn. In one case, however, a sheep which was fed the cadaver of a guinea-pig that had died of an experimental infection with one of Hamilton's strains, developed a disease and died. Of this sheep, Titze and Weichel say that it died of an "aspiration pneumonia" and showed neither the symptoms nor the anatomic changes characteristic of Bradsot. Their claim is so astounding that the details concerning this sheep must be quoted.

"Das Tier, ein 5 Jahre altes weibliches Rambouilletschaf, zeigte sich 24 Stunden nach der Infektion schwer krank: Vollständige Unterdrückung der Futteraufnahme, hohes Fieber (41, 7 C.). Dispnoe. Am folgenden Tage starb das Tier.

"Obduktionsbefund: An der Haut und Unterhaut keine Veränderungen. Im freien Raum der Bauchhöle 120 c cm. einer klaren, hellgelben Flussigheit. die beim Stehenlassen bald fest gerann. An den Vormagen und am Labmagen keine wesentlichen Veränderungen; nur die Falten der Labmagenschleimhaut waren auf ihrer Höhe leich gerötet. Dunn und Dickdarmschleimhaut waren geschwollen und leicht diffus gerötet. Die Lymphfollikel in der Schleimhaut des Darmes waren stark geschwollen. Die Mesenteriallymphknoten zeigten markige Schwellung. Die Milz war nicht verändert; ebensowenig die Nieren. Leichte Hepatitis parenchymatosa. In den Pleurasäcken 400 c cm einer blutigroten trüben Flüssigkeit, in der die verfütterten Bakterien in ziemlicher Menge nachweisbar waren. Die beiden Spitzenlappen und die beiden Mittellappen der Lungen waren dunkelrot und von leberartiger Konsistenz, auf dem Durchschnitt feucht glänzend und glatt. Die pleura über den hepatisierten Lungenpartien war mit einem 1 mm. dicken, teils gelblichen, teils blutigroten, fibrinösen Belag überzogen.

"Mit Material dieses Schafes sind Uebertragungsversuche an zwei weiteren Schafen angestellt worden.

"I. Ein drei Jahre altes Schaf erhielt ¼ liter Pleuraexudat des gestorbenen Versuchsschafes per os und bleib gesund.

"II. Ein zweites erwachsenes Schaf erhielt subcutan in der Halsgegend 10 c cm Blut von dem verendeten Versuchschafe eingespritzt, es starb nach 2 Tagen." . . .

"Der Kadaver des subcutan geimpften Schafes zeigte demnach Veränderungen, wie man sie beim malignen Oedem findet."

A glaring omission is the lack of further study of the organisms found in the first sheep, especially of the organism causative of the pneumonia. I have several strains of the vibrion septique group that regularly produce a marked lung invasion in guinea-pigs. Moreover, it is evident that certain "Bradsot characters" were present in the case of this animal. It died suddenly, it showed moderate inflammation of the mucosa of the fourth stomach and of the intestine and enlargement of the lymph glands. Titze and Weichel produced "hemorrhagic edema" in guinea-pigs with material from cadavers of partially putrified sheep that did not die of Bradsot.

They conclude that the etiology of Bradsot is entirely obscure, that the bradsot bacilli are mere cadaver bacilli, and that the diagnosis of Bradsot rests on clinical and pathologic characters.

Jensen (c) energetically attacked Miessner, and Titze and Weichel, showing that they may well have considered Bradsot, cases that were not Bradsot. that Miessner falsely based his accusations that Jensen and Nielsen had worked with old cadavers; also citing protections afforded by vaccination of sheep with bradsot vaccine.

Jensen mentions in this account that of 1,545 sheep that were fed a prophylactic dose of pure culture of the organism, 5 died of typical Bradsot; adding that such cases have also been reported from Iceland.

He gives careful descriptions of stomach linings of sheep that died of Bradsot in Iceland; these stomachs had been removed immediately after the death of the animal and placed in formaldehyd. Jensen insists that infection takes place in various foci in the mucosa that are hemorrhagic and necrotic; that the bacilli penetrate at these spots and enter the loose connective tissue of the submucosa, there multiply extensively, and then invade the whole mucosa. His illustrations seem adequately to substantiate his statements.

Jensen also quotes Nielsen (c) with regard to feeding experiments, and says, regarding the failure of workers to produce Bradsot by feeding: "Hat man z. B. dem Versuchstier den wollenen Pelz genommen? Die meisten Bradsotfälle treten ja nach der Schafschur im Herbst auf. Oder hat man das Tier in diesem unbeschützten Zustande längere Zeit hindurch einem schneidenden kalten Wind ausgesetzt und dabei gleichzeitig den Verdauungskanal, in dem das Tier sich mit kaltem oder sogar mit Reif belegtem Grass ernähren muss, einer intensen Abkühlung ausgesetz? Solche Verhältnisse eben begünstigen in der Natur das Auftreten der Bradsot, aber auch wo sie in ihren intensesten Formen wirkt, wird dennoch in grossen Schafherden oft nur ein geringer Prozentsatz der Tiere ein Opfer der Krankheit.[8]

I fully agree with Nielsen in his interpretation of the effect of cold on these infections. We know from many records of human pathology during the war (Taylor, Hartley) that any influence tending to decrease the circulation in a part allows in that part the development, even in the absence of necrotized tissue, of a gas gangrene which would never have occurred had circulation been plentiful. The blood, with its leukocytes, is in these diseases the greatest bodily defense. It is most likely, indeed, that chilling of an animal will permit a multiplication of toxic invaders which would never have occurred under normal circumstances. The factor may be looked on simply as a dilution in health, or a concentration, in the case of poor circulation, of the toxin surrounding the first invaders.

Jensen and Nielsen believe Bradsot to have its portal of entry primarily in the fourth stomach. Hamilton believes they overemphasize this conception. Nielsen's statement as to the shearing of sheep in the fall in Norway is suggestive of wound infection. It is, however, more than probable that alimentary canal infections may take place. Many infections are mentioned in which no external wound is to be found. I think that the possible minuteness of the necessary portal of entry for these organisms, whether it be external or in the alimentary canal, has been overlooked (see p. 52).

(*e*) *Black Disease.*—In New South Wales a disease of sheep exists which has been thought by some to be an anaerobic invasion. Dodd reports experimental work on this "black disease."

Black disease is very erratic in making its appearance; it is enzootic, occurring in the southern highlands of New South Wales. It appears in late summer (February) and ceases in early winter (May), usually, but not always vanishing with the disappearance of frost. Inland it is a winter disease; the winters inland are warmer than they are farther south. Animals of all ages and of both sexes are affected; the disease is apparently peculiar to sheep; the mortality is variable and sometimes heavy. Stricken animals show few symptoms, they lag behind the others, and die quickly. Necropsies of animals dying of natural infection show thin bloody liquid in the abdominal and pleural cavities and some congestion of the abomasum, occasional injection of the intestine, and injection of the skin and subcutaneous connective tissue. Decomposition appears early. Heart blood of animals found recently dead of the disease, when inoculated into normal sheep, produces local gelatinous edema, thin blood-tinged serous infiltration of the subcutaneous connective tissue, and occasionally gas. Some muscles show a dark hemorrhagic appearance not unlike that found in blackleg cases, and they have a distinct peculiar but not putrefactive odor. Other affected muscles have a clay colored appearance and on incision have a distinctly putrefactive odor. The abomasum is usually congested, and the intestines are occasionally injected, sometimes markedly so. Feeding experiments were negative. Tissue inoculations and culture inoculations sometimes killed and sometimes failed to kill guinea-pigs and rabbits, being more highly pathogenic for the former.

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[8] Did they take the fleece from the experimental animal? Most Bradsot cases occur directly after the fall shearing. And did they expose the animal in this unprotected condition to a biting cold wind for some time and thus also expose the alimentary canal of the animal, which must feed on cold or even frost-covered grass, to an intense chill? Just such conditions favor, in nature, the occurrence of Bradsot; but even where the disease is most severe it frequently happens in large flocks that only a small percentage of the animals succumb to the disease

The work mentioned above was done in 1914 with material from animals found dead of black disease and thought to have died only a comparatively short time before necropsy examinations were made. Later research showed that it was difficult to produce the disease in animals with material and cultures from sheep that had just died of black disease. Dodd says: "The conclusion to be drawn from the research work narrated is that the bacteria isolated from the blood, exudates, organs, etc., of sheep found dead of black disease, including that of the braxy type, are agonal or postmortem invaders." Heart blood, kidney, liver, mesenteric lymph gland, mediastinal lymph gland, and hepatic gland were used for these inoculations. Material from such samples killed sheep and guinea-pigs in some cases, produced local reaction in others, and failed to produce any reaction in the majority of cases. Dodd says: "It need hardly be stated that the injection of an emulsion of congested portions of abomasum or intestine would have had no useful result. The bacteria present on the (inner?) surfaces would in all probability have killed the experimental animal or produced a serious local lesion; but so would probably the injection of similar material from a normal sheep." Dodd failed to find bacteria in some of the cases on which necropsy examinations were made shortly after death. "Bacteriological workers will need no reminder that the mere finding of an anaerobic organism in the heart blood of a dead animal by cultural method, especially in such an one as the sheep, is no proof whatever that it was the cause of death, even though it proves to be highly pathogenic to certain animals upon parenteral injection." . . . "No conclusion of any value is to be drawn in connection with black disease from the cultivation and experimentation with anaerobes, isolated from animals found dead."

I fear that Dodd's later investigation was undertaken to disprove an anaerobic etiology of black disease. Dodd may be quite right in maintaining that black disease is not caused by anaerobic invaders. In that case he must look for another agent that will reproduce the picture he has given us with greater accuracy than do anaerobes. It does not seem to me that Dodd has at all disproved the possibility of anaerobic infection in his sheep cases. He has carefully avoided searching for his agent in the tissues most affected—the wall of the abomasum and of the intestine. He should have taken cultures from the external surface of these walls and grown them in standard anaerobe mediums and inoculated the cultures into sheep or guinea-pigs and found the sheep unaffected before he considered anaerobes absent in the lesions that he describes. He should also have made a careful histologic investigation of the stomach and intestinal mucosa of sheep dead of black disease and of those of normal sheep.

Attention must be called to the fact that Dodd did not study the anaerobes found in normal sheep and in those dead of black disease, that he does not give us any concrete idea of the nature of these anaerobes, and that he does not even describe the lesions produced by them in experimental animals Since when, may I ask, is no information to be derived from studying the organisms that one believes not to be the agent of the disease that he is observing? Dodd might well have isolated a few strains of pathogenic anaerobes from his black disease cases, made aggressin or vaccine from them, inoculated a large number of sheep, and found statistically whether the immunization was of value. It may be difficult in the case of such a sporadic disease as the one under consideration to do this, but Jensen's statistics are to the effect that immunization confers a considerable degree of protection against the braxy of Iceland.

Bacteria were, in my opinion, not properly searched for in Dodd's sheep cadavers. They were found in hemorrhagic and necrotic liver foci, around the margin of which they were straight, long, nonsporulating, never approaching the filamentous, usually single. Dodd believes them to be secondary, because the hemorrhagic areas that seem to be the precursors of the necrotic foci are quite free from bacteria. But liver foci are well known lesions in blackleg of cattle and are also found in gas gangrene in man.[9] It appears to me that Dodd fails entirely to conceive the toxic action of certain anaerobes.

In some of his necropsies Dodd apparently shows prejudice, or else he draws conclusions rashly. Sheep No. 34, inoculated subcutaneously behind the shoulder with 0.5 c c of exudate from the axilla of a guinea-pig, died showing local lesions and also the general lesions of black disease. The necropsy was made a few hours after death. "Abomasum: The whole of the mucous membrane was of a deep red color (probably postmortem)." . . . "Some of the muscles of the left shoulder and around the site of inoculation were dark and had a butyric odor.

"From the above it is evident that postmortem changes had occurred. This was confirmed by bacteriological examination of the various fluids and organs, as besides the bacteria of the type injected there were numerous filamentous bacilli of the malignant-oedema type and other species."

Here we see that Dodd had the truth in his fingers and failed to see it. The organism found in the original sheep killed a guinea-pig, 0.5 c c of the fluid from the guinea-pig killed a sheep with typical lesions of black disease plus the typical local lesions of a vibrion septique infection, and because of the polymorphism of the organism Dodd thought that several species were present and that postmortem invasion must have occurred.

Sheep 38 was "almost cold" when the necropsy examination was performed. "Smears from the pericardial exudate and organs showed a variety of species (cadaver bacteria)." May I protest that even in small guinea-pigs "a variety of species" of anaerobes only rarely reach the pericardium, and such a thing happens only in case the mixed inoculum is recently from earth or another heavily contaminated source? Sheep No. 38 was inoculated with an anaerobic culture from a mediastinal gland of case No. 19, which sheep was inoculated from an anaerobic culture from the hepatic lymphatic gland of case 12, which sheep died of spontaneous black disease and its necropsy examination was performed immediately after death. I should say, from long experience with the anaerobes, that it would be next to impossible to find several species of organisms in the pericardium of an animal so recently dead that it was described as "almost cold" after several successive inoculations of this sort, unless the technic employed had been gross indeed, or unless the behavior of sheep toward infection be radically different from that of cattle, hogs, man, or laboratory animals (see p. 57). Dodd did not cultivate several species from the pericardium of the sheep, and one is forced to suspect that he took polymorphic vibrion septique organisms for various species. Dodd's bacteriologic criteria are weak throughout. So far as I can see, he calls the short forms "braxy bacilli" and the long ones "malignant edema organisms." There is no mention of any careful bacteriologic study in Dodd's paper.

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[9] Med. Ress. Com. No. 39, 128

To me it is quite conceivable that black disease may be an alimentary canal invasion by a highly toxic vibrion septique type of organism that kills the sheep by its toxin and becomes septicemic only shortly before death, escaping in such small numbers that inoculation of fresh tissue not directly involved in the primary process will not reproduce the disease in some cases, whereas the inoculation of tissue that has lain for a few hours will do so in all or nearly all cases. Nielsen (Jensen, b) found that the bradsot bacilli do not always become septicemic in sheep.

We have record of cattle infections (Wulff and Laabs, see pp. 44, 45) that were diagnosed as B. Chauvoei invasions, but some of which may have been invasions of the vibrion septique group, which show no muscle lesion at all. We have record of hog invasions by strains of the vibrion septique group, in which no external lesion was to be discovered and muscle lesions were absent, and in which the resemblance to braxy was marked. It is to be expected that material from such cases when inoculated subcutaneously or intramuscularly into the thigh of an animal will produce edema and sometimes gas at the site of inoculation. But Dodd's organisms and Gilruth's organisms and also Titze and Weichel's organisms reproduce, on inoculation, also the injection of the abomasum and of the intestine and other lesions produced by the disease in its natural form. It is to be expected that in the case of artificial subcutaneous or intramuscular inoculation death will occur before the mass infection in the alimentary canal is as great as in the case of internal infections. The injection of the membranes will then also be less than in the case of an alimentary canal invasion. Vibrion septique strains produce strong toxins, and when an infection is under way, it is a matter of only a few hours before death ensues.

Dodd is right in holding a skeptical attitude in studying a disease of this sort; he is to be commended for choosing fresh cadavers for his investigations. He is quite right in saying that the finding of a highly pathogenic anaerobe in the tissues of a dead sheep is no proof that that anaerobe was the cause of the death of the sheep. But the finding of a highly pathogenic anaerobe in the tissues of an animal not long dead demands careful investigation as to its location and quantitative distribution in the body of that animal before one is warranted in stating that the anaerobe was not the cause of the death of the animal. I feel that Dodd entirely underrates the pathogenicity of the organisms of the vibrion septique group, and confounds them with "cadaver bacilli." This is easily understood because of the prevailing confusion on this point. It must be emphasized that the highly pathogenic anaerobes are members of definite restricted groups that are few in number, and that there are a vast number of species of anaerobes that may be called "cadaver bacilli" and have no invasive power of their own. Lumping all anaerobes together as "cadaver bacilli" because most of them are putrefactive is as primitive a procedure as would be the lumping together of all aerobes for a similar reason.

In questioning the anaerobic origin of black disease Dodd is also forced to question the anaerobic etiology of Gilruth's nonwound infection braxy cases. This he has a right to do as Gilruth's observations are not founded on sufficient data to be conclusive. He also questions the anaerobic etiology of the northern braxy. I feel that it would be necessary for him also to question, on the same ground, all anaerobic infections of cattle in which no external lesion is to be found—which, according to Wulff, is true of the vast majority of blackleg cases. The parallelism between the anaerobic cattle and hog infections and these sheep diseases is close.

Grosso identifies the bacillus of Bradsot with the malignant-edema bacillus by means of the agglutination reaction.

Zeissler (a) states that the bacillus of Ghon and Sachs is the cause of Bradsot in sheep, goats, and hogs, including wild hogs. He and Eugene Fraenkel found the bacillus of Gohn and Sachs in Bradsot in a sheep and a goat. I presume that the statement regarding hogs is based on the work of Köves (see p. 81). Zeissler asserts that the bacillus of Gohn and Sachs is the one specific cause of Bradsot.

(f) The Epidemiology of the Anaerobic Infection of Sheep and Tentative Conclusions as to Such Infections.—We know that anaerobic invasion of sheep by organisms of the vibrion septique group and of the blackleg group may take place after wound contamination. This phenomenon is entirely analogous to that of cattle infection.

Other infections of sheep, which do not follow the contamination of visible wounds, do not present a patent parallel to cattle infections. The striking differences are two: First, anaerobic infections of sheep are usually caused by organisms of the vibrion septique group and rarely caused by organisms of the blackleg group, and anaerobic infections of cattle are usually caused by organisms of the blackleg group and less frequently by organisms of the vibrion septique group. Second, the infections of sheep are more often localized in the alimentary canal and serous lining of the peritoneal cavity, while in cattle the infective process is more frequently localized in the muscles. Granted that the predominating invaders of cattle, namely, the organisms of the blackleg group, prefer muscle tissue, and the predominating invaders of sheep, the organisms of the vibrion septique group, prefer somewhat the serous membranes, I do not believe that this fact alone explains the great number of alimentary canal localizations in sheep. There must either be a constitutional difference between sheep and cattle in this respect, or there must be a difference in the mode of infection in the two species. It seems to me that a satisfactory explanation of the differences would be covered by two considerations: first, the acceptance of Nielsen's and Jensen's contention that alimentary canal infection is common in sheep; second, the consideration that if insects may transmit these diseases, cattle would be far more exposed to infection of skeletal muscle than sheep, for sheep may be bitten by free flying insects over a very small portion of the body. The fact that Bradsot is common in winter and blackleg is common in summer fits in closest harmony with these considerations. Moreover, on inoculation, blackleg is highly pathogenic for sheep, and vibrion septique strains are highly pathogenic for cattle, and a difference in the susceptibility of the two types of organisms for these two types of transmission may explain the discrepancy in the predominance in natural infection of one group in cattle and of the other in sheep.

Data concerning sheep infections are not sufficient to permit a discussion of insect transmission. We may, however, consider more closely the question of alimentary canal invasion. Jensen's evidences as to the mode of invasion in the Bradsot of Iceland are probably sufficient, though the reason for such a mode of invasion is unknown. In Germany and in other countries, however, where the lesions are not so uniform, other factors must be considered. The fact that intramuscular inoculation of vibrion septique strains into guinea-pigs and sheep produces gastro-enteritis makes necessary the careful search for a minute portal of entry in bradsot cadavers. This has been pointed out by Hamilton and by Gilruth. The suggestion that organisms of the blackleg group do not show the preference for growth on the abdominal serous membranes that is shown by strains of the vibrion septique group would be worth considering in the case of suspected Bradsot with slight alimentary canal lesions. Vibrion septique strains also vary somewhat among themselves in respect to production of gastro-enteritis in the guinea-pig.

I hope that after the publication of this article it will no more be stated that because bradsot material was capable on experimental inoculation of producing "malignant edema," Bradsot cannot be considered to be caused by an anaerobe. No one who has studied the action of anaerobes in the animal body would for a moment hesitate to declare the possibility that Bradsot may be an alimentary canal localization of the disease known as "malignant edema." These diseases are analogous to the group caused by one specific organism: B. anthracis. The diseases caused by this organism are known as malignant pustule, woolsorter's disease, and intestinal anthrax—depending on the site of the multiplication of the organism, and on the mode of transmission and inoculation. Infections of the subcutis and muscles by organisms of the vibrion septique group are called "malignant edema"; infections of the fourth stomach and intestine of sheep by organisms of the vibrion septique group are known as "braxy" or "Bradsot"; vibrion septique infections of the lungs and pleural cavity are termed "pneumonia." It is time that more emphasis were placed on the specific etiology of diseases and less on the lesions produced by them.

The chaotic condition of the subject of the anaerobic infections of sheep and the diseases that resemble them is, to my mind, due to several causes. First, although the disease of braxy has long been considered an infection, many of the investigations concerning it have been undertaken from the pathologic-anatomic point of view. Second, when infections have been considered probable, the idea of a specific causative organism has been held to and few cases have been properly studied. Third, the epidemiologic rules applied to infectious diseases in general have been applied also to these diseases, which differ from ordinary infections in that they are caused by accidental invasion by organisms that are frequently present in the alimentary canal of the host: organisms to which the host usually shows immunity. Therefore mechanical, physical, and chemical factors must be considered as inducing alimentary canal invasions by anaerobes, and the closest analogues to such invasions are to be found in wound infections and not in contagious infections. Common sense and not dogma should be our guide in seeking the etiologic elements concerned in these diseases, which certainly cannot be included in those infections that must be judged by the four rules of Koch.[10] Feeding experiments have amply proved that these diseases are not transmissible diseases in the ordinary sense. Certain factors to be considered in anaerobic alimentary canal infection are:

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[10] Koch himself says as to the epidemiologic considerations involved in wound infections (a, 75): ". . . ein vollgültiger Beweis . . . nur dann geschafft werden kann, wenn es gelinkt, die parasitischen Mikroorganismen in allen Fällen der betreffenden Krankheit aufzufinden, sie ferner in solcher Menge und Verteilung nachzuweisen, dass alle Krankheitserscheinungen dadurch ihre Erklärung finden, und schliesslich für jede einzelne Wundinfektionskrankheit einen morphologisch wohl characterisierten Mikroorganismus als Parasiten festzustellen.

"Sollte es denn nun aber möglich sein, diese Bedingungen überhaupt jemals zu erfüllen?"

(100) "Denn je länger ich mich dem Studium der Infektionskrankheiten befasst habe, um so mehr habe ich die Ueberzeugung gewonnen, dass das Generalisieren neuer Tatsachen hier verfrüht ist und dass jede einzelne Infektionskrankheit oder Gruppe nahe verwandter Infektionskrankheiten für sich erforscht werden muss."

1. Gross wound infection in a normal animal. The mucosa being cut by a foreign object or deeply pricked by a sharp point, may become contaminated by a toxin producing organism, and wound infection is far more likely to occur than in the case of an infected external wound of the same size for two reasons: the conditions are more anaerobic, and the infective agent probably is usually present in the vegetative form.

2. The infection of a minute wound in a debilitated animal—such a case as that suggested by Nielsen in which freshly shorn sheep are turned out in the cold. Also animals sick from some other cause may come under this head: Kitt's cases of hogs dying of Schweinepest and anaerobic invasion (see p. 81) would probably come under this head. Perhaps also Meyer's cases (b) of cattle with liver foci, which were apparently caused by organisms of the vibrion septique group that were nonpathogenic to cattle on artificial inoculation. I believe that abundant cases of this kind might be collated from human necropsies.

3. Infection of wounds either in the alimentary canal or in the liver; these wounds are caused by flukes or other parasites (worms are common in sheep). This possibility has been emphasized by Gilruth.

4. It is quite conceivable that organisms of the toxic type may sometimes find suitable food material so abundant in the alimentary canal that they multiply to such an extent that their toxin overcomes the general resistance of the wall of the alimentary canal and they invade en masse. The "bacterial felt" pictured by Jensen (c. plate 3) is suggestive of such a condition, and Jensen has always held this form of invasion to be the usual one. Debilitated animals would of course be most likely to succumb to such toxic assault. One may well conceive, however, that in a healthy animal in which some intestinal stasis occurred, or in one in which some highly concentrated suitable food were present for the rapidly multiplying anaerobes to grow on, an unusual multiplication of organisms might occur, and invasion might, in isolated cases, take place.

5. Some chemical agent in food (for example, poisonous plants) may break down the resistance that the animal tissues offer to the toxins and aggressins of the organisms.

6. The ingestion of earth may, as suggested by Gilruth, promote anaerobic alimentary canal invasion.

A final criticism of the research performed on this subject is in place. To my knowledge, a careful, thorough investigation either of the mixed flora or of pure strains of the anaerobes involved in the alimentary invasions of sheep has never been made. Elaborate technic for such study has not existed, but even such technic as has existed has not been applied. The study of the anaerobes is difficult and time consuming and the anaerobic field is enormous. The veterinary pathologist cannot alone hope to solve the problems involved, unless he become bacteriologist as well and spend many long months in the study of the organisms with which he experiments. Unless the investigator has time for such study, experimental research on this complicated problem will lead nowhere. It is a problem to be undertaken seriously by a laboratory having ample funds and many experimental animals and by a worker who is able to devote his full time to the subject. If he have the services of a trained epidemiologist, so much the better. Fragmentary inoculation experiments with organisms of whose nature and relative distribution we know nothing lead us to confusion instead of to knowledge.

3. REINDEERPEST

A discussion of this disease must not be omitted.

Lundgren first reported it in detail in 1898. He brought with him from Lapland several tubes of pericardial and peritoneal and pleural fluid from a reindeer on which he made a necropsy examination. This material served for an elaborate and painstaking investigation by Bergman, who published an account of his work in 1901.

So far as these two publications show, there has been reported by an experienced pathologist only one necropsy examination on a reindeer supposedly dead of the spontaneous form of the disease, namely, Lundgren's case. Bacteriologic investigation is reported on material from this single case. Identification of this case with the other cases of the diseases known as reindeerpest rests entirely with the Lapps. The Lapps are a somewhat primitive people of exceedingly keen observation and scientific perception; especially, as Bergman says, may they be depended on to report with accuracy observations concerning reindeer. But I do not think that Lundgren and Bergman are justified in concluding that the pathogenic organism which was evidently the cause of the death of this reindeer is the one etiologic agent involved in the causation of reindeerpest or even in calling it the bacillus of reindeerpest.

Reindeerpest is a disease of the summer time and of the valleys. Reindeer are kept for most of the summer in the mountains on account of insects, and if they are brought down to the valleys they may die in large numbers of reindeerpest. When the herd is taken again to the uplands the disease disappears, when brought down again it reappears, only to die out in September. Epidemiologically the disease differs markedly from the diseases already discussed in only one respect, namely, that a large number of animals are attacked in a herd. There are three possibilities in its epidemiologic classification: that it is a contagious disease, or as Lundgren suggests, that it is an insect-borne disease, or that it is a disease introduced through abrasions or wounds, which diseases are probably governed in their ability to infect by several factors that may vary in different localities. Lundgren notes, for example, that during the summer when reindeerpest was most prevalent there was also a great deal of hoof disease among the animals, and he suggests that the pest originates as an infection of the diseased hoofs that partakes of the nature of a wound infection. The conditions in Lapland are different in many ways from those farther south. If it is true that the incidence of the disease ceases when the animals are driven into the mountains, the contagious nature of the disease is thrown into doubt, thus making it less likely that it is of the hemorrhagic septicemia type. Insects are numerous during the summer in the valleys of Lapland, and the reindeer is one of the animals most persecuted by them. The incidence of the disease in valleys and its occurrence in summer resembles the incidence and occurrence of blackleg. Valleys are highroads and are muddy; insects are there most abundant. The Lapps are an inquiring people.

Bergman says that they often perform a necropsy examination on their dead reindeer to find out the cause of death, and they keep and use the skins and let the bodies lie. The latter conditions favor anaerobic infection. As to the natural mode of infection of this disease, nothing is known. Young animals are the victims, even very young ones.

Reindeerpest runs an acute or peracute course. Symptoms when observed are: restlessness, an irregular gait, lagging. The eyes protrude, they are inflamed; the animals stop eating and ruminating; they are thirsty; breathing becomes difficult and rapid; they may cough and have a bloody discharge from the nostrils; sometimes swellings appear on various parts of the body. The sick animals have a disagreeable odor. Lundgren's necropsy findings agree with accounts of necropsies related to him by the Lapps. They are so astoundingly similar to those narrated in cases of anaerobic invasion that I shall quote them in abbreviated form: The animal was so swollen that the skin was nearly bursting. The legs on the upper side stuck out straight. The swelling covered the entire subcutis; the nasal apertures were filled with a bloody, foamy liquid. The whole subcutis was filled with small gas bubbles and a little liquid. The muscles and fat were sprinkled with blood. The blood was black and clotted. In the pleural cavity were two liters of clear hemorrhagic fluid, in the pericardial cavity a similar liquid—paler and not so clear. The pleura was injected; the heart muscle contained numerous small gas bubbles. Other lesions are mentioned. The peritoneum was vividly injected, with occasional branching extravasations. There was a large amount of serous transudate, like that in the pleural cavity. The mucous membrane of the first stomach was normal, that of the abomasum was diffusely red colored. In the intestines, which contained little gas, there were occasional branching extravasations. There was no postmortem discoloration. The liver and spleen contained gas. The kidneys were soft and contained gas. A reindeer inoculated with material from this animal showed more gas formation, and differed in its lesions from the above in details only; it had an unpleasant odor.

Bacterial Studies: Four of the five samples brought to Bergman by Lundgren were contaminated by mold. Bergman reproduced with them in various animals a disease in most ways similar to the disease in the reindeer on which Lundgren performed necropsy examination. From the organs of these animals and from the transudates of the original animal Bergman invariably grew an aerobe that was a facultative anaerobe. This aerobe grew in colonies on the surface of gelatin plates. Bergman describes it minutely. In culture it grew abundantly in the form of heavy rods 1.6x0.8 mikron, and reluctantly formed spores. It was a gas and acid former and grew on all mediums. It resembled several strains of sporulating aerobes that I have encountered. In the animal body the morphology of the organism, as shown by Lundgren's photographs and by Bergman's photographs, is most astoundingly like that of an organism of the vibrion septique group. Moreover, Bergman's bacillus forms long chains on the peritoneal surface and liver surface of animals. His account of the mode of spore formation of his bacillus is also characteristic of that of a vibrion septique type of organism.

Bergman's bacillus was highly pathogenic for reindeer, sheep, guinea-pigs and white mice, and it was also pathogenic for a calf, cats, brown rats, pigeons, and sparrows, and for frogs at 27 degrees. It was not pathogenic for rabbits, hogs, dogs, and chickens. I would suggest that there are not many species of organisms known that are so widely pathogenic. Bergman immunized sheep with sublethal doses of his organism. This animal was not immune to blackleg.

The pathogenicity of Bergman's material decreased rapidly in liquid cultures and not quite so rapidly in cultures on solid mediums. Pathogenicity could be recovered by animal inoculation.

We have here before us two possibilities: First, An aerobic organism exists (and we know of no other such aerobes) that is most strikingly similar (in morphology, incidence, pathogenicity, and in the lesions that it produces) to the organisms of the vibrion septique group, but which rapidly loses pathogenicity in aerobic cultures. Second, Bergman had to do with a symbiotic mixed infection of an organism of the vibrion septique group and an aerobic sporebearer, the latter able to render conditions suitable for the growth of the former on a plate. One hesitates to suggest the second contingency, for Bergman was evidently a careful worker and himself tried to demonstrate the absence of such a possibility; but it seems to me that such a possibility cannot be ignored. Bergman does not tell us of a careful examination of anaerobic colonies, or whether anaerobic cultures lost their virulence rapidly. He tells us of a dried gelatin plate with colonies, individual ones of which were nonpathogenic. "But if there were in the whole plate only one colony that contained virulent reindeerpest bacilli, that would be sufficient to infect a reindeer." So he washed off the plate and inoculated the suspension into a reindeer and produced the disease.

I should suggest that if the losses from reindeerpest are considerable today, it would be worth while to study many cases of the disease, or at least several cases from various valleys. Investigation of the diseases of reindeer is, however, according to Lundgren, an extremely arduous pastime. Bergman's work is interesting and a step in the right path, but little can be done with material from one specimen. It must be borne in mind that anaerobic invasion may at times be a fatal one secondary to another type of infection.

4. THE ANAEROBIC INFECTIONS OF THE HORSE

I believe that earlier reports tend to diagnose horse infections as blackleg more frequently than do later ones.

Rivolta, in 1881 and 1882, described an organism that he isolated in 1870 from a horse and named Bacterium cuneatum. It was pathogenic for horses, hogs, and rabbits. He later mistakenly identified it with B. Chauvoei. I quote from Arloing, Cornevin, and Thomas, and am unable to tell whether Bacterium cuneatum may or may not have priority over Pasteur's organisms.

Hutyra and Marek (p. 46) state: "In horses the occurrence of blackleg has not been definitely determined. The literature contains reports by Gantner, Hafner, Schmitt, Vemelage, Fischer, and others, but, in these cases, a confusion with pseudo-blackleg or malignant edema is not only not excluded, but even probable. Hasenkamp, for instance, found in a horse a serous-hemorrhagic fluid in the thoracic cavity and pericardial sac, hemorrhages in the serous membranes, and black, crepitant, softened muscles in the shoulder region, the neck, and the hind leg, which were also infiltrated with a sero-sanguinolent fluid. Quadecker observed in a similar case a sweetish odor in the diseased parts which was neither pleasant nor foul."

Arloing, Cornevin, and Thomas (86) failed repeatedly to produce more than a local swelling in horses and asses by inoculating blackleg virus.

Gantner reports the death of a horse in a place where Rauschbrand of cattle was frequent. The animal was much depressed, vomited, and had dyspnea. Necropsy showed bloody gelatinous edema, gas infiltration on neck and breast, hemorrhages in the muscle, and spleen tumor. The causative organism was Rauschbrand as established by Hafner.

Foth (b, p. 212) reports the death of a foal that died in a blackleg-infested meadow. Shoulder muscle and breast muscle were black. Foth, who is a critical worker, was unable to distinguish the anaerobic invader from true blackleg. Wulff (b), working in Foth's laboratory, later denied the occurrence of Rauschbrand in horses. Foth failed, as did Arloing, Cornevin, and Thomas, to produce blackleg in the horse by artificial inoculation.

Ostertag declares that blackleg never occurs in horses.

Veterinary statistics in Germany annually record Rauschbrand in horses, usually from two to seven cases per year.

Levens reported Rauschbrand in a horse from the neighborhood of Cleve. Symptoms were carefully described. The disease was evidently localized in the muscles and not in the alimentary canal. Some lesions were found in the heart and the lungs were hyperemic. The causative organism was half the length of a red blood corpuscle, spores were median or terminal and the organisms killed guinea-pigs and not rabbits. Levens complained that the name "Pseudo-rauschbrand" has no place in veterinary terminology; the case was typical Rauschbrand and the organisms were typical Rauschbrand.

Wulff (b) reproved Levens for his diagnosis, explaining that his criteria were insufficient. He stated that all horse and hog material that has come to his laboratory with Rauschbrand-like appearance contained no Rauschbrand bacilli but edema bacilli, and that all efforts to produce infection of horses and hogs by means of inoculation of Rauschbrand material have failed. From the work of Foth and of Wulff in Schleswig it is evident that there must be a great preponderance of true Rauschbrand infection over vibrion septique infection in cattle. But we know that in other parts of the world, even in Germany, vibrion septique infections occur in cattle, and we know that such infections are usually termed Rauschbrand. It is easy to understand Leven's assurance in proclaiming his horse case Rauschbrand when we realize that cattle infections of the same nature are regularly diagnosed as Rauschbrand.

Jensen and Sand reported malignant edema in the horse as a wound infection in 1886.

Von Werdt (a, p. 839) states that the horse is the domestic animal most susceptible to malignant edema (the proteolytic organism).

Hutyra and Marek (p. 34), in referring to malignant edema, say: "For natural infection of domestic animals solipeds are most susceptible." It appears to me, however, that horse infections cannot be common, or there would be more references to them in the literature.

Friedberger and Fröhner (p. 182) state that they have seen malignant edema in horses after injury to the tongue, after tooth extraction, and after castration. Friis reports an infection in the horse after an injection of eserin. Other authors quoted by Friedberger and Fröhner have described malignant edema in the horse.

Zschokke describes a case of malignant edema in the horse, following a nail wound in the hoof. The nail wound had healed and the fatal infection appeared nine days after wounding.

Bambauer reports the death of three horses, which was due to anaerobic infection. The organism was not Rauschbrand and not malignant edema; it was pathogenic for mice and for guinea-pigs, and not for rabbits.

Schlemmer reports an infection in a colt that he studied closely. The causative organism was pathogenic for guinea-pigs, rabbits, mice, rats, and pigeons, was nonproteolytic, formed filaments on the surface of the liver of animals, and formed woolly colonies. It was identified by him with the bacillus of Ghon and Sachs.

Weinberg and Séguin (b, p. 292) describe an infection by a vibrion septique strain that was being used to immunize a horse. A centrifuged toxin was injected intravenously into the unimmunized horse, and an infection resulted that was first evident as an edematous swelling of the neck and right foreleg and chest. Crepitation appeared later. Vibrion septique antitoxin was administered in large doses, and the animal overcame the vibrion septique infection, only to succumb to secondary infection in the large area of necrotic tissue. The invading organism is included in my collection under the name HV.

Diedrichs reports the investigation of material from two horses that died of a Rauschbrand-like disease.

The material from the first horse produced what were apparently mixed infections fatal to guinea-pigs and pigeons. Cultures failed later to kill guinea-pigs. The culture was identified as Pseudorauschbrand in the Hygienic Institute of Berlin. The second strain remained proteolytic through several inoculations; but a guinea-pig. inoculated with a culture that had had several animal passages had little putrefactive odor and showed a colorless edema as thick as a finger; no gas is mentioned. The mixed (proteolytic) culture killed rabbits and was somewhat pathogenic for pigeons. It would seem that oedematiens-group infection is not improbable in this case. Infections of the vibrion septique group are probably never accompanied by proteolytic organisms through several passages. They do not produce edema as thick as a finger, and they produce gas. According to Diedrichs, the organism was not blackleg, and the pathogenicity tests apparently bear him out.

Von Hibler reports no cases of anaerobic infection in horses. He isolated B. oedematis maligni "im sinne von Koch" (Von Hibler X) from a mule.

Weinberg and Séguin report (b, p. 320) the case of a mule used for making antivenin. As a result of the venin inoculation a mixed infection of the head and neck developed. Edema was extensive, as was the tissue destruction, the latter being almost unbelievable in its extent. The infection was apparently due to B. histolyticus and B. oedematiens. The animal recovered after inoculations of mixed serum.

No samples of material from horses had come to this laboratory until Dr. Wood was so kind as to furnish me with four specimens, which were derived from horses that were being used for an immunity experiment. The pathogenic strain from one of the four horses was a typical vibrion septique: of interest is the fact that the animal, which had developed an extensive edema, recovered spontaneously from the infection.

The three other samples contained an interesting organism. Careful tests showed the three strains to be alike. The infected horses all succumbed, showing on section edema and gas formation. This organism (strains O 55, O 51, O 09) behaves much as do oedematiens strains. Inoculation of 0.5 c c of 24-hour meat medium culture of this bacillus into the thigh of a guinea-pig may kill the animal in 18 hours or less without any invasion of the body by the bacilli, or if little toxin be present the infection kills in 24 hours with septicemic invasion. A white gelatinous edema, that does not rapidly lose its gelatinous consistency on section, is found to surround the inoculated region. It is slightly blood stained. No gas formation takes place. A marked injection of the abdominal serous linings is the notable internal lesion.

On meat medium the organism produces a little gas, occasionally turning the meat pinkish, but producing so little acid that the pink color soon fades. The short, rather heavy bacilli are scarce in the medium, they stain palely as a rule, do not sporulate actively, and in 48 hours many individuals show the peculiar ghost-like appearance characteristic of oedematiens strains. Auto-agglutination takes place. The growth on milk is like that of B. oedematiens: reluctant and slow, very little acid being produced. On blood broth H_2S is produced, otherwise proteolysis is not patent. In colony formation, however, this organism is definitely to be distinguished from all the oedematiens-group strains in my collection.

Christiansen (a) found that normal horse serum protects guinea-pigs against infection with B. Welchii.

I think that it can be stated that horses are subject to infection by organisms of the vibrion septique group and that they are subject to infection by organisms of the oedematiens group. I do not believe that any case of true blackleg infection in the horse has been established.

5. ANAEROBIC INFECTIONS OF THE HOG

It is apparent in the case of hog infections, as it was in the case of horse infections, that early diagnoses were more generally referred to blackleg and more recent diagnoses have been referred to the organisms of the vibrion septique group.

Arloing, Cornevin, and Thomas (p. 89) failed on repeated trials on all types of hogs to produce blackleg by inoculation. They state (p. 90) that hogs are susceptible to gas gangrene.

Hutyra and Marek (p. 34) state that hogs are rarely affected by malignant edema, and they say (p. 43) that attacks of blackleg have so far been observed only in fattening establishments.

Marek and Battistini have described a few cases of Rauschbrand in hogs. The symptoms in Marek's cases were pharyngeal angina and lameness. Section showed great swelling of the neck and submaxillary regions and of the affected legs, serous edematous infiltration of the muscles and peripharyngeal tissues and dark reddish-brown coloration and crepitation of the muscles. The causative organism was identified as Rauschbrand. Three rabbits inoculated with it died, but infected muscle did not infect sheep or a hog on inoculation. Marek believes that the infection took place through the superficially injured mucous membrane of the pharynx or through the injured tonsils. He says that Rauschbrand is not uncommon in hogs, although it is not epidemic.

Willenberg reports a case of Rauschbrand in the hog. It was so diagnosed on bacteriologic examination. There was much gas in the subcutis and in the muscles and liver. "Ausgeprägte Fäulnisserscheinungen" were evident shortly after death, but what these were cannot be gathered from the review.

Balás reports a case of Rauschbrand in the hog. The case may safely be assigned to the vibrion septique group because the organism killed a guinea-pig in 15 hours with emphysematous swelling at the site of the inoculation.

Paukul demonstrated malignant edema in hogs; a crepitating swelling developed from a wound in the skin.

Oppermann speaks of malignant edema in the hog; the disease follows castration and other wounds.

Kitt (e, p. 290) mentions 2 cases of wild hogs that were wounded by the tusks of others, which, on necropsy, showed lesions remarkably similar to those of Rauschbrand. Later Kitt (f) found gas gangrene in 3 animals dying of Schweinepest. The organisms causing this condition were whetstone and snowshoe shaped clostridia and ordinary rods. There was gas in the liver, which showed long chains and clostridia. There were hemorrhagic-diphtheritic stomach and intestinal lesions. Only one of the hogs was wounded, and Kitt believes that the infection was an intestinal invasion that occurred when the animals were already seriously debilitated by Schweinepest. The organism was not, however, on inoculation into normal hogs, pathogenic for them. It was pathogenic for guinea-pigs and rabbits. It was, according to Kitt, neither malignant edema nor Rauschbrand.

Köves reports 26 cases of hog infection that have come to his notice in the past 6 years. The causative organism is the same in all cases, though there are slight differences. Clinically the disease is divisible into two groups: (1) the group in which the skin and subcutaneous muscle are affected, and in which the infection much resembles Rauschbrand, and (2) the group in which the stomach wall only is affected and in which the appearance is similar to Bradsot. In these cases the mucous membrane of the stomach lining may be thickened to 2 or 3 mm. and be covered with dirty yellow diphtheritic deposits. The submucosa are infiltrated with reddish liquid. All cases gave an organism which is identified as the bacillus of Ghon and Sachs and is not malignant edema or Rauschbrand.

K. F. Meyer (a) reviews this subject. "The organism isolated from hogs by Marek, even though morphologically identical with B. chauvaei, had a high pathogenicity for rabbits; the publications of Marek do not contain any detailed account as to the biochemical actions of the observed organism. The same remarks apply to the publications of Born and Battistini in which, based purely on morphologic similarity of the observed organisms in the diseased muscles, the diagnosis of symptomatic anthrax in hogs was made." Meyer reviews the transmission experiments of von Ratz and holds that the lesions described by von Ratz so closely resembled those produced in hogs by the bacillus of Ghon and Sachs as identified by himself that he doubts, in the absence of cultural studies, whether von Ratz was really working with true symptomatic anthrax. He calls attention to the work of Glässner and of Wulff, who attempted to infect hogs with blackleg material and failed to produce more than a local swelling. Meyer says: "I cannot therefore agree with v. Ratz that hogs are susceptible to symptomatic anthrax."

Meyer reports the occurrence in Pennsylvania of a disease of swine that broke out in a concrete piggery in which all the animals had been immunized against hog cholera two months previously. "Four out of sixty animals died, all with symptoms suggestive of hog cholera. Only one was autopsied. It showed gas-phlegmon of the pharyngeal region of the neck; acute serous pharyngitis and laryngitis; foamy liver; pulmonary edema." An anaerobe and B. suipestifer were isolated from the spleen, liver, kidneys, and tonsils. The anaerobe was identified as the bacillus of Ghon and Sachs. The same anaerobe was found in the muscles of another of the four dead hogs; the organism forms filaments on the liver of animals, is pathogenic for hogs, guinea-pigs, mice and pigeons, and sometimes for rabbits and rats. Agglutination tests showed it to be neither B. Chauvoei nor the malignant edema bacillus of Koch. The strain differed in minor details from a vibrion septique strain sent to Meyer from the Pasteur Institute, but agreed closely with the description of organism No. I described by Ghon and Sachs.

Dr. Haslam has sent me a culture from one of several hogs that died of an anaerobic infection. The organism is a typical strain of the vibrion septique group.

Recently Proescher and Hoffman described an outbreak of malignant edema in hogs which were being inoculated for the manufacture of hog cholera serum.

Von Hibler reports a case of infection in a wild hog that was caused by B. Novyi.

The lesions resembled those of Rauschbrand. Von Hibler was in possession of Novy's original strain of B. oedematis maligni II. This strain and others resembling it do not in my hands produce in the guinea-pig an infection remotely resembling blackleg. B. oedematiens Weinberg, strain Joly, does, however, produce a black hemorrhagic edema, as black as that produced by blackleg organisms. B. oedematiens and B. oedematis maligni II. are closely related and should logically be placed in the same group. It would be interesting to examine the strain isolated from the hog by von Hibler. One must remember that a hog and a guinea-pig might show different lesions in the same infection.

Von Hibler also isolated B. Welchii (B. phlegmones emphysematoseae) from a hog that was thought to have died of pest.

I think it may be stated that hogs are susceptible to infection by organisms of the vibrion septique group, especially when they are weakened by other diseases, or when they are wounded. I do not believe that there is only one such invader that can be termed specific.

The question of invasion of hog tissue by organisms of the blackleg group is to my mind still an open one. These organisms are in general less pathogenic than those of the vibrion septique group. To prove anything in regard to this point a number of indubitable strains of the blackleg group of differing characters should be used. Probably exhaustive study would show that strains of the blackleg group are distinctly less pathogenic for hogs than strains of the vibrion septique group, and are incapable of infecting sound tissue of healthy animals, but that, were a wound severe enough, a blackleg strain would be capable of penetrating hog tissue, as was the Welch strain in von Hibler's case. The organisms of such wound infections should be looked on as relative in their pathogenicity; their action depends on so many factors that they cannot be considered in the light of our conceptions of ordinary infectious diseases.

Oedematiens group organisms are pathogenic for hogs (von Hibler).

B. Welchii (B. phlegmones emphysematosae, Fränkel) may be pathogenic for hogs. Von Hibler does not state how his hog may have contracted the infection.

6. ANAEROBIC INFECTIONS OF THE DOG

Hutyra and Marek (p. 34) state that dogs and cats are rarely affected by malignant edema.

Friedberger and Fröhner (p. 182) mention having seen malignant edema in dogs, which developed as a result of bites, and also as a result of operations that were presumably performed with soiled instruments.

Livesey observed malignant edema in a dog.

Charrin and Roger found that subcutaneous inoculation of infective fluid from guinea-pigs that had died of a vibrion septique infection produced in dogs a local edema, little gas, necrosis and eschar formation. The process was never fatal, produced mild general symptoms and usually conferred immunity.

Evidently dogs and cats are more highly resistant to anaerobe infection than are herbivores, or we should hear more of such infections. It must also be borne in mind that carnivores are far more agile and skilful animals, and in the domesticated state are probably far less subject to wounding than are the herbivores.

7. AN EXPERIMENTAL INFECTION IN A CAMEL

An experimental infection in a camel is reported by Cross. He inoculated with blackleg virus a 7 year old camel and 2 yearling camels. All died of the infection. The only lesion found was a large emphysematous swelling at the seat of inoculation. The muscles of this part were of a dark brown to a black color and of a slightly rancid odor. Spontaneous outbreaks of the disease in camels are said not to have been reported.

8. ANAEROBIC INFECTIONS OF GAME

Olt and Ströse state that malignant edema is rare in game animals and that Rauschbrand has not been reported in game animals.

Budd, in 1863, quotes Hintermeyer who described in 1846 an outbreak of "quarter evil" in a herd of deer in a park in Germnay. There was an exudate from the nose, anus, and vagina of the animals. Just what the disease was we can probably not determine.

9. ANAEROBIC INFECTIONS OF RODENTS

Guinea-Pigs: I have observed two cases of spontaneous infection in the guinea-pig, which were caused by organisms of the vibrion septique group.

Von Hibler reports B. phlegmones emphysematoseae and B. VII. and B. tetani as infecting a guinea-pig inoculated with earth. I have observed a fatal B. Welchii infection in a guinea-pig that had received an intravenous inoculation of B. typhosus. Guinea-pigs are highly susceptible to many anaerobes existing in earth.

Pasteur and Koch early used guinea-pigs for the isolatoin of pathogens from earth.

Gaffky studied an infection derived from earth that was pathogenic for rabbits and not for guinea-pigs.

B. Novyi and B. tetani were found by von Hibler in a rabbit that was inoculated with earth. This sort of infection could be described a thousand times—it is not highly significant. Injection of earth into many guinea-pigs and mice and rabbits would give a collection of many diverse tissue-invading strains. Koch largely used mice for the study of malignant edema.

I believe that, in general, mice, rats, and rabbits are less susceptible to anaerobic invasion than are guinea-pigs, but this may be merely an impression. Cornevin found the guinea-pig most susceptible to experimental anaerobic infections.

10. WHALE SEPTICEMIA

In 1888 Nielsen (b) discovered and described a septicemia of whales that was due to wound infection. The natives of a region near Bergen caught whales by shooting them with infected arrows and then waited a day or two for the infection to develop before harpooning the animals. Nielsen described the causative organism of this infection and declared it to be similar to (but not identical with) the causative organism of blackleg. Recently M. Christiansen (b) has made a close study of an organism isolated by him from several samples of muscle from a single whale, which were taken in the late eighties. He places the organism in the same group as the bacillus of Ghon and Sachs.

for which he proposes the name "Ghon-Sachs group," and he shows various characters differentiating his organism from B. Chauvoei. Morphologically and pathogenically Christiansen's organism is distinctly to be placed in such a group, in spite of the fact that he insists that it is not flagellate, which character he believes to be an unimportant one. I have myself isolated an organism from whale muscle, which was kindly furnished me by Dr. C. O. Jensen. Dr. Christiansen states that my strain is identical with his strains; they all come from the same whale. I find that my strain is definitely referable to the vibrion septique group, but it is markedly distinct from any other strain in my possession. I should be much surprised if only one species were involved in all these wound infections of the whale. Very likely, however, one species was more pathogenic for whales than others and so became the predominant or more usual invader.

Obst found in 287 swelled cans of sardines an anaerobic organism "in pure culture." Assisted by W. G. Smillie she identified the organism as B. wallfischrauschbrand Nielsen. No animal experiments are recorded, and no standard anaerobe mediums were used in this study. The organism formed gas from protein and carbohydrate mediums; a foul odor was present in broth cultures. It sporulated heavily, forming "round" spores, giving the bacillus a "tennis racket formation." I can see no possible justification in thus identifying an anaerobe. It is counter to all chances that the anaerobic organisms in the 287 cans were in pure culture in the cans, and that the organisms in the 287 cans were the same. There is probably ample justification for identifying the bacillus of Christiansen with that of Nielsen. This organism is not proteolytic and forms oval spores. The aerobist should be warned that there are a thousand chances to one that a stray proteolytic anaerobe picked up from anywhere has not been described. Moreover, the possibility of any accurate identification of such an organism from a printed description is slight. The most we are today justified in doing when we wish to identify an anaerobe is to determine its group affinities. A careful and detailed description of a pure culture of the organism is then in order. Anaerobic literature is in no condition to be referred to for specific identifications of more than a scant handful of organisms; an authoritative systematic work on the subject does not exist.

11. NONMAMMALIAN ANAEROBIC INFECTIONS

Birds seem to vary in their susceptibility to anaerobic infections. Fowls are usually or invariably reported as immune. Pigeons are apparently subject to artificial infection with some strains of the organisms of the vibrion septique group but not to blackleg; they are highly susceptible to Welch bacillus infection. Sparrows may be killed by some vibrion septique strains.

Arloing, Cornevin, and Thomas (p. 91) produced infection by blackleg in frogs that had been kept at 22 C. in water. The organism of Bergman (see p. 75), which is not proved to be an anaerobe, infected frogs kept at 27 C. in air.

12. THE NOMENCLATURE OF ANAEROBIC DISEASES

It has probably been noted that the subject of the terminology of anaerobic infections is in an exceedingly chaotic condition. This is primarily because human and veterinary clinicians and pathologists have frequently attempted to diagnose the diseases seen by them according to the causative organism that they imagine is involved in a given case, instead of describing the disease as they find it and sending properly collected specimens to the bacteriologist for examination. Moreover, it is my experience that an opinion based on clinical and microscopic findings early in the examination of material from a case, is likely to prove mistaken or incomplete. Far too often has the bacteriologist, who found in a gas gangrene sample the ubiquitous B. Welchii, contented himself with this finding and searched no farther.

Ghon and Sachs, who carefully reviewed the subject of nomenclature in 1903-04, came to a very simple conclusion, which, were the facts as they then appeared to be, could be accepted as a basis of terminology. Their idea was to term "malignant edema" the disease caused by the Bacillus oedematis maligni (under which name they identified Koch's, Pasteur's, and their own organism) and to call "malignant emphysema" the disease caused by Bacillus phlegmones emphysematoseae, Fraenkel. They state that this system has its shortcomings, but they do not wish to introduce more terms into the nomenclature.

Not to introduce new names when new conceptions arise would be hyperconservative; but it would seem also that the definition and application of old names needs revision. The whole field of anaerobic invasion must first be glanced over. We have a number of diseases to consider, which vary:

1. According to their location in the animal body: subcutis, muscle, pleural and peritoneal linings, glandular organs, alimentary canal.

2. According to their mode of inoculation: wound infections demonstrable as such, and infections whose point of entrance is obscure.

3. According to their distribution from the site of inoculation: through lymph or blood channels, through muscle or connective tissue spaces, or along lining surfaces.

4. According to the specific nature of the host, its age, and its condition.

5. According to the primary group affinities of the invaders: proteolytic, nonproteolytic, or mixed proteolytic-nonproteolytic.

If the infection is nonproteolytic, it varies:

6. According to the species of the invaders involved: (I have good reason for believing that wound infections, at least, are far more frequently and more highly polyspecific than they are usually thought to be), or according to the species of the one invader.

7. According to the relationship between the toxicity, the aggressive powers, and the reproductive powers of the particular strain or species of the principal invader, on which depend edema production, gas production, transudate formation, muscle invasion and hemolysis.

The varieties of specific invaders may be thought of as moderately numerous.

It is, according to the above desiderata, quite ridiculous longer to chain any specific invader to any pathologic term. Such an effort would be futile and would continually embarrass the pathologist. What we need is a set of purely pathologic diagnostic terms; descriptive terms we have in plenty. We may use old names for the pathologic processes encountered whenever they are descriptive of the lesion to which they are applied; but in so doing we must discard entirely the implication of the expression of an opinion as to the identity of the organism causing the lesion. The human and veterinary pathologists sorely need such a system as this whenever the material that passes their hands is to be examined by a bacteriologist. The pathologist cannot possibly be expected to determine the specific cause of an anaerobic invasion. He must have not only the descriptive terms that he possesses, but also diagnostic terms that do not in any way commit him to specific or even generic bacteriologic determination. The identity of such terms is for the pathologists to decide on. The bacteriologist is then free to make a diagnosis of the group or specific affinities of the invading organisms. I propose, however, that the term malignant edema be given to the pathologists to abandon or to use as they see fit, and the term "bacillus of malignant edema" be discarded forever from bacteriologic terminology. The name B. oedematis maligni will also have to be abandoned unless someone, somewhere, can produce a culture whose history can be traced to Koch's laboratory, which reproduces, on subcutaneous inoculation into laboratory animals, the lesions described in "Zur Aetiologie des Milzbrandes." No literary identifications of other strains with that of Koch's description can scientifically be accepted. Adamson's proposal to use the name B. oedematis maligni for the sporogenes type of organism has no systematic precedent, is illogical, and is likely to increase the existing confusion in our terminology.

The use of the term "gas bacillus" is likewise to be discouraged because it has become more or less definitely associated with B. Welchii, which is no more a gas bacillus than are the vibrion septique organisms.

The anatomic diagnosis of "Rauschbrand" or "blackleg" should, in my opinion, for legal purposes, include infections by members of the blackleg group and of the vibrion septique group, or else some other term should be devised to include both types. There is no logic in separating the two diseases and recompensing the farmer for cattle dying of one and not of the other; moreover, different local veterinarians will probably never agree in their diagnoses of these diseases. The bacteriologist's diagnosis should depend on the organism or organisms found, and the two workers, pathologist and bacteriologist, can thus work in harmony.

I do not think that we are as yet in a position to take a stand as to the terminology of the infections of sheep. There should be a term (and Bradsot and braxy probably fill the bill) that is descriptive of a rapidly fatal disease of the alimentary canal, which is associated with abundant multiplication of anaerobic organisms in the tissue. No specific or even generic meaning should be implied by the expression "anaerobic organisms." "Bradsot" or "braxy" may be taken to apply to such a disease in sheep or in goats or in hogs, as Zeissler has used it, or in other animals as well (see cattle, p. 45). When a thorough investigation of the obscure cases in sheep has been made it will be time to revise the nomenclature of such diseases.

GENERAL CONCLUSIONS

For more detailed conclusions see pp. 4, 5, 30, 31, 35, 45, 47, 54, 72, 74, 77, 80, 83 and 87.

Cattle are subject to spontaneous infection by organisms of the blackleg group and, somewhat less frequently, by organisms of the vibrion septique group, both of which types of infection are usually diagnosed as "blackleg."

Sheep are subject to spontaneous infection by organisms of the vibrion septique group and somewhat less frequently by organisms of the blackleg group. Both types of infection are probably diagnosed at times as braxy, as blackleg, and as malignant edema. It is possible that other diseases that are not of anaerobic origin are at times diagnosed as "Bradsot" or "braxy."

The possibility exists that reindeerpest, as described by Lundgren and Bergman, is an anaerobic infection.

Horses are subject to infection by members of the vibrion septique group. Such infection may or may not follow a wound. It was formerly frequently diagnosed as blackleg. Horses are also subject to infection by organisms of the edematiens group. True blackleg group infection in the horse has probably never been demonstrated.

Hogs are subject to infection by members of the vibrion septique group. Such infection was formerly generally diagnosed as blackleg but has more recently been diagnosed as "malignant edema," "Ghon-Sachs bacillus infection," "specific gas phlegmon of hogs," or "Bradsot," depending on the location of the process. Hogs have probably never been shown to suffer from spontaneous blackleg infections.

The comparative rarity of oedematiens-group infections in animals, except perhaps in the horse, and the great rarity of serious invasion of animal tissue by B. Welchii are to be noted.

In general many species of herbivorous mammals are subject to spontaneous anaerobic infection, both following and not following demonstrable wounds, and apparently the animals most susceptible are ruminants, cattle and sheep, and possibly reindeer. Carnivores and man are subject to anaerobic infection only when wounded or when seriously debilitated by sickness.

All immunization work in connection with anaerobic diseases depends on a recognition of the group (see pp. 4 and 5) affinities of the anaerobic organisms infecting the animals of the particular district in which immunization is proposed.

The factors concerning anaerobic infection that are of most interest, namely, the epidemiologic factors, are almost wholly unexplained, and their demonstration would prove a most valuable contribution to science and to agriculture.

I wish to make a plea for the world-wide study of these infections. They cost every agricultural country vast sums every year, and notable steps in the direction of immunization have shown that such immunization is feasible. To decide definitely the mode of infection in these diseases and the incidence of the group and specific entities involved would be of immense value to any country and to the world at large. The specificity of the toxins and of the aggressins of the different members in each group must be investigated. There are a hundred interesting immunologic and epidemiologic problems to be suggested. The proper investigation of the subject can be done only in a laboratory that is well equipped to handle anaerobes and that has the funds to employ a trained epidemiologist who knows something of veterinary pathology and can spend all his time in the field.

My most heartfelt acknowledgment is due Dr. Karl F. Meyer for his leadership, his encouragement, and his instruction. His wide fund of information in the field of animal pathology, his many suggestions as to the bibliography of the subject, his resource in general bacteriology, and his generous interest in the work of a student, all have been of the greatest value to me during the prosecution of my anaerobic studies.

I wish also to extend my hearty thanks to the workers who have been so kind as to aid me in making my collection of strains.

APPENDIX A

CATALOG OF MATERIAL INVESTIGATED

MATERIAL FROM CATTLE, ALL OF WHICH HAD AT SOME TIME BEEN DIAGNOSED AS "BLACKLEG"

VIBRION SEPTIQUE GROUP STRAINS (QUOTATION MARKS INDICATE THE DESIGNATION OF THE SENDER)

BX96—"Blackleg Sierraville." Dry shreds of muscle from cow. Given me by Dr. Meyer. Strain BX was also isolated.

BX—"Accession 775." Blackleg muscle from animal inoculated with piece of calf muscle taken from what was considered to be a typical case of blackleg. Strain 775 was also isolated. Received from Dr. Edward Records of Reno, Nev.

BM—"Blackleg Mohler." Calf muscle from Bureau of Animal Industry Strain BMB was also isolated. Given me by Dr. Meyer.

BF1—"Blackleg 4460." Brain culture from Dr. O. M. Franklin, Wichita, Kan.

BF4—"Blackleg NKax." Brain culture from Dr. O. M. Franklin.

BC11—"Blackleg Calif. 11." Brain culture, pink. Given me by Dr. Meyer.

BC5—"Blackleg Calif. 5." Brain culture received from Dr. Meyer.

BK—"B. anthracis symptomatici 'Muscelstück.'" Agar stab, received through kindness of Dr. W. Frei, Zurich, Switzerland.

BT—"B. anthracis symptomatici III, Plaut." Agar stab received through the kindness of Dr. W. Frei.

BQ—"Rausch?" Gelatin shake, received from Dr. Theobald Smith.

BP—"B. Chauvaei." Old desiccated blood broth culture. The strain of Leclainche and Vallée, which Dr. Meyer received in egg-broth culture from the Pasteur Institute.

BRH—"Reliance." Agar shake from Dr. Haslam, Sioux City, who did not diagnose it as blackleg. He states that it came from one of several calves killed by a pellet vaccine that was presumably thought to be a blackleg vaccine.

AS4—"B. anthracis symptomatici 4." Agar slant, strain mentioned on p. 53.

BLACKLEG GROUP STRAINS

BX—"Blackleg Sierraville." Same sample as BX96.

BC3—"Blackleg Calif. III." Dry shreds of muscle from cow. Given me by Dr. Meyer.

BL—"San Jose." Muscle from steer on Miller and Lux Farm near San Jose. Received from Dr. Meyer. This was a typical case of blackleg. The liver of the animal showed infarcts and was foamy.

BMB—"Blackleg Mohler." From the same sample as BM.

BEI—"I." Muscle from calf from Dr. A. Eichorn of Pearl Harbor, N. J.

BEII—"I." Muscle from calf from Dr. A. Eichorn.

BEIII—"III." Muscle from calf from Dr. A. Eichorn.

BEIV—"IV." Muscle from calf from Dr. A. Eichorn.

BEF—"V." Muscle from calf from Dr. A. Eichorn.

BF2—"Blackleg 585x." Brain culture from Dr. O. M. Franklin.

BF3—"Blackleg 377E." Brain culture from Dr. O. M. Franklin.

BF5—"Blackleg C538." Brain culture from Dr. O. M. Franklin.

BDDVII—"B. Chauvoei VII." Agar shake from Parke, Davis and Co.

BDDIII—"B. Chauvoei III." Agar shake from Parke, Davis and Co.

BD—"Blackleg." Muscle from cow. Received from Dr. Schalck of North Dakota.

BB—Suspected blackleg material from calf. From Dr. Traum, University of California.

VA—Material from guinea-pig inoculated from calf that succumbed to blackleg. Furnished by Dr. J. Traum of the University of California.

775—"Accession 775." Blackleg muscle from animal inoculated with piece of calf muscle taken from what was considered to be a typical case of blackleg. Received from Dr. Edward Records of Reno, Nev. Same sample from which BX was isolated.

801—"Accession 801." Blackleg muscle from a calf, received from Dr. Records.

MATERIAL WHICH FURNISHED NO PATHOGEN

BS—"B. Chauvoei." Agar shake.

BA—"B. Chauvoei." Agar shake.

SA—"Bacillus anthracis symptomatici strain 60." Agar slant.

BK—"Agar 517 Rausch Kral." Gelatin shake.

BH—"B. anthracis sympt. Schnürer." Agar stab received through the kindness of Dr. von Frei.

BV—"B. anthracis symptomatici V," "Friedenburg. Hartl." Agar stab. Secured for me by Dr. W. Frei.

B203—"B. anthracis sympt. No. 203 Kl. Schierstadt. Grosso." Agar stab. Secured for me by Dr. W. Frei.

AS2, 3, 6, 7, 10, 12, 25, 49, 61—Marked "B. anthracis symptomatici." Agar slants. All contained anaerobes.

Material from Sheep, All of Which Had at Some Time Been Diagnosed as Bradsot

VIBRION-SEPTIQUE GROUP STRAINS

RS—"Bradsot Schierstedt, Halle." Muscle from sheep, received from Dr. C. O. Jensen of Copenhagen.

RO—"Bradsot Opsta Norway." Muscle from sheep, received from Dr. C. O. Jensen.

RR—"Bradsot Rostock." Muscle from sheep, received from Dr. C. O. Jensen.

RL—"Bradsot Halle I." Muscle from sheep, received from Dr. C. O. Jensen.

RH—"Bradsot Halle III." Muscle from sheep, received from Dr. C. O. Jensen.

RNL—"Bradsot Sletten Norway." Muscle from sheep, received from Dr. C. O. Jensen.

RNS—Same sample as RNL. These two strains differ entirely in colony characters, by which means they were separated, and they differ in the lesions that they produce in the guinea-pig.

BJP—"Bradsot bacillus of Jensen." Brain culture given me by Dr. Meyer, who received it personally from Dr. Jensen in 1913.

BLACKLEG GROUP STRAIN

RG—"Bradsot Graf Bülow Halle." Muscle from sheep, from Dr. C. O. Jensen.

SAMPLES FROM WHICH NO PATHOGEN WAS ISOLATED

RB—"Bradsot Faroe Oeerne." Muscle from sheep, received from Dr. C. O. Jensen.

RA—"Bradsot Anhalt, Tierarzt Schlemmer." Muscle from sheep, received from Dr. C. O. Jensen.

RP—"Bradsot Scotland, Prof. Hamilton." Muscle from sheep, received from Dr. C. O. Jensen.

STRAIN DIAGNOSED AS BLACKLEG

RM—Received from Dr. Hadleigh Marsh, Montana. See p. 60.

Material from Horses

VIBRION SEPTIQUE STRAINS

HV—Meat cultures of serous exudate from horses accidentally infected with centrifuged toxin. Weinberg and Nicholas, C. R. Soc. Biol. 1917, 80, p. 709. This was a strain of human origin.

HG—Brain culture given me by Dr. F. W. Wood of Berkeley. The organism was isolated from an extensive edematous process in a horse. The animal recovered.

OEDEMATIENS GROUP STRAINS

O 09, O 51, O 55—Brain cultures given me by Dr. F. W. Wood. They were isolated from three horses that succumbed to an extensive edematous and emphysematous process.

Material from Hogs

VIBRION SEPTIQUE GROUP

GG—"Ghon-Sachs bacillus." From guinea-pig material infected with the hog bacillus of Dr. Meyer (Jour. Infect. Dis., 1915).

PS—"Sioux City." Agar shake from Dr. Haslam, who isolated the organism from a hog that was one of several that died of the same disease.

Material from Guinea-Pigs

VIBRION SEPTIQUE GROUP

GPX—Spontaneous infection in a guinea-pig. From this laboratory.

580J—Spontaneous wound (?) infection in the guinea-pig. From this laboratory.

OEDEMATIENS GROUP

NO—"B. oedematis maligni II." Type strain received from Dr. Novy in blood culture. Jour. of Hyg., 1894. Now true to type culturally and morphologically, but nonpathogenic.

MATERIAL WHOSE HISTORY I AM UNABLE TO TRACE, BUT WHICH MAY BE REFERABLE TO SMALL ANIMALS

VIBRION SEPTIQUE GROUP

MP—"Vibrion-septique Pasteur." Strain given me by Dr. Meyer, who received it from the Pasteur Institute in 1915.

VS—"Vibrion septique Pasteur." Strain given me by Miss Robertson, who received it from Dr. Roux. It is not the same species as MP, and is unique in my collection in several respects.

MATERIAL SIMILAR TO THE ABOVE, FROM WHICH NO PATHOGEN WAS ISOLATED. SOME CULTURES ARE OMITTED

OH—"B. oedematis thermophilis, Novy." Agar stab received from Dr. Frei. Proteolytic.

MS—"B. oedematis maligni, MS. Plaut." Agar stab received through the kindness of Dr. Burkhardt, who received it from Kral's museum.

MK—"B. oedematis maligni, Koch." Agar stab received through the kindness of Dr. Burkhardt, who received it from Kral's museum.

KO—"B. oedematis maligni, Koch." Received through the kindness of Dr. Frei.

MATERIAL FROM WHALE

VIBRION SEPTIQUE GROUP

WS—"Hvalseptikaemie." Material from whale, collected by Nielsen (?) in the late eighties and sent here by Dr. C. O. Jensen; see p. 85.

APPENDIX B

SUMMARY CLASSIFICATION OF THE STRAINS LISTED

VIBRION SEPTIQUE GROUP STRAINS: 30

An attempt to classify these according to type would be premature.

From cattle, 13: BP, BM, BCII, AS4, BF1, BC5, BX96, BQ, BT, BF4, BR, BK, BX.

From sheep, 8: BJP, RXS, RXL, RS, RO, RH, RR, RL.

From horse, 2: spontaneous infection: 1, HG; accidental laboratory infection: 1, HV.

From hogs, 2: GG, PS.

From guinea-pigs, certain 2: GPX, 580J.

Probably from guinea-pig or rabbit, 2: VS, MP.

From whale, 1: WS.

BLACKLEG GROUP STRAINS: 21

From cattle, 19. Typical 15: BX, BC3, BL, BEII, BEIV, BEIII, BF2, BF3, VA, 775, BD, BDDIII, BDDVII, BB, BF5. Atypical, probably none identical, 4: BMB, BEF, BEI, 801.

From sheep, 2: Typical (probably) 1: RM. Atypical, 1: RG.

OEDEMATIENS GROUP STRAINS: 3

From horse: O09, O51, O55.

CULTURES AND MATERIAL THAT YIELDED NO PATHOGEN

From cattle, 16: BV, B203, BH, SA, AS2, AS3, AS6, AS7, AS10, AS25, AS49, AS61, BS, BA.

From sheep, 3: RF, RP, RA.

From guinea-pig, 1: NO.

Source unknown, probably from laboratory animals, 4: KO, MS, MK, OH.

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PRINCIPLES CONCERNING THE ISOLATION OF ANAEROBES

STUDIES IN PATHOGENIC ANAEROBES. II

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The subject of the isolation of anaerobes is one which the worker is inclined to approach with apologies. Every month or so a paper appears in some journal in which a new and expeditious procedure for the separation of anaerobes is described. There are many successful ways of isolating anaerobes and it is unwise to recommend any one method above all others. I have succeeded with various arrangements; and wish in this paper to analyze some of the principles governing the isolation of these organisms and to explain a few of the pitfalls which have caused many workers to believe that the securing of "absolutely pure" anaerobic cultures is a difficult matter. With a little practice and with the exercise of much discrimination, anaerobes may be isolated as quickly, or nearly as quickly, as aerobes.

Contamination occurs somewhat more frequently in anaerobic cultures than in those of aerobes. Contamination of originally pure cultures may be attributed to the following causes: (1) Insufficiently sterilized media; anaerobe media are usually pasty and require more careful sterilization than others. (2) Inoculation transfer involving the exposure of the cotton plug and of the inoculum to the air. I have noted in working in London and near the sea in San Francisco, that the more dusty the air, the more frequent are contaminations, and the contamination flora may vary according to location. (3) During incubation in closed jars the cotton plugs may become sufficiently moist for molds to grow through them; where a mold can grow a bacillus can follow. (4) During prolonged incubation water of condensation may even run into the tubes from the top of the jar. (5) If stored in closed cans molds may grow through the plugs. Workers should take these points into consideration in planning their work. Anaerobic jars are exceedingly convenient and practical for periods of incubation under four or five days, and for much anaerobic study twenty-four to forty-eight hours incubation is sufficient. Prolonged incubation should be made under vaseline or in the case of sugar-free media in exhausted sealed tubes. Sealing of tubes is inadvisable where carbon-dioxide may be so confined that it produces an acid end-point. Re-incubation of cultures in exhaust jars should be cautiously undertaken so that the medium may not boil up to the cotton plugs. Anaerobic jars which do not require exhaustion are preferable for re-incubation of cultures.

The commonest contaminators of my cultures have been cocci and molds, not anaerobes. The reason that anaerobic contamination of anaerobe cultures is so very common probably lies principally in the uncritical handling of such cultures. If a coccus or mold contaminates a culture the worker immediately kills such an organism, but if an anaerobe enters the tube it proceeds to multiply unmolested. Daily watchful observation of the cultures studied is absolutely necessary for successful anaerobic work. I have not found indications of any so-called symbiotic tendency that makes anaerobes more difficult to isolate than aerobes.

Anaerobes vary greatly in their behavior and requirements, and the method of isolation must be adapted to the problem in hand as it turns up. Each combination of two or more species of organisms presents different elements for consideration and for adaptation of technique. There is no one method that is always best, and it is only after a worker knows something about the nature of the particular organisms that he is dealing with, their cultural behavior, and their morphology in the medium in which he regularly grows them, that he is able quickly and surely to isolate numbers of strains.

It is, of course, desirable to make use of methods that may be applied to the largest possible number of species, that are easy of manipulation, and moderate as to cost of time and material.

The organisms present in material to be investigated may belong in any one of four large groups, which may be described as follows:

ORGANISMS	UNDESIRABLE	DESIRABLE
Easily killed by heat	Non-sporulating aerobes, common, many species	Non-sporulating anaerobes: Welch bacillus is, in most media, the chief consideration
Not easily killed by heat	Sporulating aerobes, not common in pathological material; species numerous, however	Sporulating anaerobes, species legion in number

Whatever be the material that is to be investigated, a microscopic examination of a Gram stain is first in order. Practice only will enable the worker to form judgments which will be of value to him. As hints to the beginner, one may suggest that there are an endless number of species of anaerobes and that specific diagnosis by microscopic examination is futile. There are frequently many species of anaerobes in the material that finds its way to a laboratory, and, unless a study of many strains is intended, the isolation or demonstration of a single species, whose nature is guessed at, must be attempted. If the microscope shows the probability of the presence of that species, matters are simplified. To seek a certain organism one should familiarize himself with a pure strain of that type of organism, or study photographs or drawings of it; verbal descriptions are not of much value. He should also learn the colony form of *several strains* of the type he desires to obtain. The employment of a medium in which the morphology of the organisms is varied and characteristic is imperative. This laboratory uses chopped meat medium containing 5 per cent peptic digest broth (pH 7.2) for routine cultivation and this medium excels all other autoclaved media in the above respect. The use of oil over the medium to produce anaerobiosis should be avoided whenever possible for routine work, as it interferes with the making of satisfactory smears; for long incubation and under certain circumstances demanded by technical considerations, vaseline will be found very useful. Ghon and Sachs recommend the use of agar for stratification; liquid media should be frozen before the agar is poured.

Heating. To free sporulating organisms from non-sporulating organisms heating is always resorted to. Heating of inoculum may be performed in one of two ways. Heavily inoculated media may be heated to 80° in a water bath for fifteen to thirty minutes. This method is highly inaccurate, especially in case pasty media are used, but it serves on occasion. Or the material to be inoculated may be heated in a Pasteur pipette after the following fashion:

Sera, exudates, and muscle extracts should be diluted with sterile saline. Cut the end of a Pasteur pipette off square with a file, flame it, then draw up the inoculum for about two inches by capillary attraction, and seal the pipette with less than a quarter of an inch of air space between the tip and the liquid. To kill non-sporulating organisms heat in a waterbath for ten minutes at 70° to 72°. Then flame the pipette above the inoculum to kill organisms that may have been above the water-line, mark the tip in several places with the file or diamond, slowly flame the tip, insert it in the tube of fresh medium, flame a pair of light forceps and with them break the tip of the pipette against the inner wall of the tube and expel the material.

If a worker is certain that the type of sporulating anaerobe desired is always highly resistant to heat, he may use higher temperatures, in the neighborhood of 100°C. for heating his cultures. Dr. K. F. Meyer informs me that he has repeatedly employed this method with success in the isolation of *Bacillus botulinus*. Von Hibler sowed mixtures containing such organisms, and even less resistant ones, directly into hot agar. Some strains of *B. botulinus* and of Novy's bacillus are highly resistant to heat.

1. To segarate non-sporulating anaerobes from aerobes

1. Heat to 56°-58° for five or ten minutes. This occasionally serves the purpose.

2. Try to induce sporulation by growing the mixture on alkaline sugar-free medium, such as alkaline egg, or serum medium (von Hibler, 1908, p. 189). When the anaerobes form spores, heat. This procedure is a sure method of freeing *B. Welchii* from ordinary aerobes: incubate for four days. This organism is found in a sporulating condition in soil and in fecal material.

3. Try a pathogenicity test. If the organism sought is pathogenic it may be recovered in pure culture from the animal tissues. Use this method for *B. Welchii*, *B. egens*, *B. fallax*.

4. Use selective media. For the Welch bacillus use milk or 1 per cent glucose broth. Inoculate it with a pipette, a fresh tube of medium every twelve hours if possible.

5. Use good anaerobic methods. Cultivate the material on meat medium in strict anaerobiosis, inoculate in agar dilution tubes that have been thoroughly boiled, and fish the colonies. This technique is described on page 461.

6. Northrup suggests the use of a 25-cc. burette, in which the organisms of an inoculated mixture will, on short incubation, sort themselves out, the aerobes growing above, the anaerobes below, where they may be drawn off through a stopcock.

II. To separate non-sporulating anaerobes or reluctantly sporulating anaerobes from other sporulating anaerobes

1. Use selective media, milk, with short incubation periods, for *B. Welchii*.

2. Use animal inoculation.

3. Use shake cultures.

4. Use semi-anaerobiosis: The non-sporulating anaerobes are naturally more resistant to oxygen than the sporulating ones.

Aside from *B. Welchii* this sort of organism is rarely sought after or noticed. Few non-sporulating anaerobes are described, and the group has been generally neglected, but careful methods show that non-sporulating anaerobic rods and cocci are not uncommon.

III. To separate either variety of anaerobes from sporulating aerobes

Sporulating aerobes are rather infrequently found in pathological material. One meets them frequently, however, in a medium that has been insufficiently sterilized. My encounters with sporulating aerobes have been so rare that it would be wise to recommend that a worker always go back to the original material and test it for the presence of any sporulating aerobe that he finds in a culture with which he is working. Avoid sporulating aerobes, do not contaminate cultures with them, and isolate the anaerobes from the original material again.

1. Sporulating aerobes are of two classes: strict aerobes (any good anaerobic technique followed by a colony method will free a culture of these) and facultative anaerobes. I have never encountered a sporulating facultatively-anaerobic aerobe that grew better under strictly anaerobic conditions than its accompanying anaerobes. Any strictly anaerobic colony method that will separate anaerobes from each other will separate them from aerobes. In my experience trouble with abundantly growing aerobic organisms denotes faulty anaerobiosis: the presence of a small amount of oxygen that permits the undue multiplication of the aerobes. My experience has, however, been almost entirely with pathological material and I may have failed to meet with the most troublesome aerobic organisms.

2. Kitasato and Weyl found that anaerobes were less sensitive to pyrocatechin, chinon, sodium formate, and sodium sulphindigotate than were the aerobes causative of cholera, typhoid and anthrax. Rivas continued this type of investigation.

3. Churchman has investigated the inhibitive effect of gentian violet on aerobic growth. Hall recommends the use of gentian violet in a dilution of 1:100,000 to separate sporulating aerobes from anaerobes. This, I should think, would work very well for the heavy Gram positive organisms of the *B. subtilis* group, provided the desired anaerobe is not of the same nature.

4. The spores of aerobes may sometimes be satisfactorily germinated in broth in a Petri dish, the broth being then heated and inoculated into agar.

IV. To separate sporulating anaerobes from non-sporulating anaerobes and aerobes

Heat as described on page 448.

V. To separate sporulating anaerobes from other sporulating anaerobes by cultural methods

I. *Heating.* The following diagram shows how heating may be employed:

ANAEROBIC BACTERIA	PROTEOLYTIC GROUP	NON-PROTEOLYTIC GROUP
Early sporulating species (18–24 hours)	Bifermentans group *et alii.* Do not occur very frequently	Nearly all sporulating organisms
Later sporulating species (24–48 hours)	Sporogenes group *et alii*	
Late sporulating species (48 hours on)	Tetanus group, botulinus group, *et alii*	

This diagram shows that if proteolytic early-sporulating organisms are absent, as is frequently the case, a saccharolytic form may be isolated or be rendered relatively far more abundant by heating eighteen to twenty-four-hour cultures successively. I have had mixtures of *B. sporogenes* and organisms of the blackleg group that were not pathogenic for guinea-pigs because of the scarcity of *B. Chauvoei.* Two successive heatings and inoculations made blackleg the predominant organism and the culture was highly pathogenic. This method is also excellent for organisms of the vibrion-septique group and for many non-pathogenic saccharolytic bacteria, as well as the early-sporulating proteolytic ones.

II. *Selective media.* Isolation methods usually depend on securing a predominance of the organism sought. To increase the relative numbers of an organism with whose nature one is familiar, a medium should be selected on which the organism grows best. For saccharolytic species mixed with proteolytic ones, use sugar-containing media. Meat medium plus 1 per cent glucose is good, meat medium not neutralized in the making is also good. Ordinary meat medium, the culture being taken early in its development, is usually sufficiently selective. Sugar media selective for certain groups may be used, if the number of cultures to be isolated warrants the investigation of the sugars split by that group. I have found that culture in casein-digest liver-broth renders blackleg the most able guinea-pig invader in a blackleg-vibrion-septique mixture. To increase the percentage of proteolytic organisms use meat medium or brain medium in a culture two to four days old, or even older; or employ the medium of Achalme-Passini, salt solution or broth containing cubes of egg-white; or use serum medium or other sugar-free media; or a medium made up at pH 8.0 or above. For an organism whose morphology interests one and whose nature is not known, experiments should be tried with various media, and the behavior of the mixture should be studied. Under laboratory conditions certain types always tend to disappear from mixed cultures. It must be kept in mind that conditions must exist in nature which favor the multiplication of such species or they would have died out long ago. For such organisms try media of vegetable origin.

In taking samples of pathological material enrichment with the tissue in which the organisms are found is advisable. Schottmüller isolated septicemic streptococci in blood-glucose agar shakes. The many tissue-containing media favor the growth of pathogens. (Media summarized by Pfuhl.) Tunnicliffe used serum and ascites agar for the anaerobic coccus found by her in measles cases. Plotz and his co-workers added ascites or hydrocele fluid to glucose agar for blood cultures from their typhus patients. Dick and Henry employed blood-glucose agar for the various anaerobes found in the blood of scarlet fever patients. Leucowicz used serum-sugar agar for *Fusiformis*.

Digest media are excellent for anaerobes. A number of such media are discussed by Stickel and Meyer.

Serious problems sometimes arise. Thus, *B. tetani* is particularly difficult to isolate from gross mixtures, as it is not a tissue invader, and because it sporulates later than the organisms that usually accompany it. In case an organism like *B. tetani* grows excellently on a given medium but its accompanying organisms grow better than it does, try similar selective media of modified reaction, or make use of exhaust media of the type recommended by Tulloch. One may always grow the objectionable species or several species in a medium till growth ceases, filter the medium and then grow the mixed culture in the filtrate. In case this fails one may add a minute quantity of some solid protein for a starter. Tulloch added a bit of rabbit kidney to an exhaust filtrate and found it highly selective for *B. tetani*. Von Hibler grew mixtures containing *B. tetani* on clotted rabbits' blood and stated it to be selective for that organism. But his photographs of the organism show his cultures to have been so badly contaminated that he may have been mistaken.

I have found a modification of a medium of Beijerinck's excellent for the enrichment of soil anaerobes (sodium phosphate 0.05 per cent, ammonium sulphate 0.05 per cent, soluble starch 1 per per cent, calcium carbonate 0.5 per cent). The anaerobic flora obtained in such a medium after heating a soil emulsion is very different from that obtained in meat or other media of complex composition. By fishing large lenticular or modified lenticular colonies from 2 per cent agar shakes of this medium which have been incubated for four days, the large butyric acid bacteria of the genus *Clostridium* may be isolated with comparative ease. Winogradsky (1902) recommends the use of media free of fixed nitrogen for the isolation of nitrogen fixing anaerobes (*Clostridium Pastorianum*); this medium is described by Fred (1916) and Bredemann used it for the isolation of his *Bacillus amylobacter* which he considers to be the same organism as Winogradsky's. Milk may also be used as an enrichment medium for many organisms of this genus.

Omeliansky (1904) describes the following method for enriching cellulose fermenters: Place in a long-necked flask any cellulose substance, paper, cotton, flax; add chalk, and fill to the top with water which contains 0.1 per cent ammonium phosphate, 0.1 per cent calcium phosphate, 0.05 per cent magnesium sulphate, and a little sodium chloride. Inoculate with slime or horse manure, cover, and set in the dark. In other publications (1895;

1902) he gives other formulae; several are given by Fred. Ankerschmitt used physiological salt solution containing cubes of potato to enrich splitters of hemicellulose. Choukevitch employed 1 per cent pepton broth with 5 per cent starch for starch splitting organisms. Silicate jelly as a substrate for such of these organisms as will not grow on agar is described by Omeliansky (1899) and formulae for similar jellies are given by Fred and by Küster.

III. *Symbionts* have been used to enrich certain types of anaerobes. Sturges and Rettger found that *B. putrificus* flourished best in the presence of *Bact. coli*, and used the latter as a symbiont for the former. Rhein used *Bact. faecalis-alcaligenes* as a symbiont for anaerobes, cultivating them in the presence of air: this organism has several advantages. Wilson and Stere describe a cocco-bacillus which is an excellent anaerobe symbiont.

IV. Another resource is to test the *resistance* of the desired species to *unfavorable circumstances*. Thus McCoy and Bengtson of United States Public Health Laboratory isolated many strains of tetanus with great ease by heating toxic strains at 70° for a half hour and inoculating the spores in veal agar dilution shakes. This technique is adverse for an anaerobe, but *B. tetani* appears to be hardy enough to withstand it. Modified highly acid or alkaline media, or media poor in protein may be used for such purposes. A pure strain of the desired organism is invaluable in testing out media of this sort.

V. *Aniline dyes* may be used to eliminate certain species of organisms and the possibilities which they offer are almost unlimited.

VI. *Selective temperatures* may be employed for enrichment of various organisms. *B. botulinus* was long thought to produce toxin at low temperatures only, because the contaminating organisms in the cultures outgrew it at 37°. Thermophilic organisms are of various types, and are discussed by Bergey. Major W. J. Tulloch tells me that the flora obtained by incubating a mixture of anaerobes in meat medium at 42° is quite different from that obtained at 37°, slender, oval end-sporing organisms predominating. It is probable that anaerobic organisms will be found that grow at much higher temperatures than at 42°.

VII. *Separation of organisms before sowing* was suggested by Stoddard, who shook his material with sea sand to separate encapsulated or autoagglutinated organisms. Dr. K. F. Meyer tells me that he has found such technique useful in isolating anaerobes from soil and from old meat cultures which had sporulated heavily. Such separation is not necessary when fresh cultures are used.

VIII. *Isolation by various colony methods.* Because of the confusion that exists as to the purity of cultures of anaerobes, it will be well to study the biological factors involved in the genesis of bacterial colonies. A colony is an aggregation of organisms that are prevented from mixing with other organisms by a physical obstruction. A colony may be defined as follows:

a. From one single organism—the ideal colony for isolation purposes.

b. From two or more organisms descended directly or indirectly from one organism—a satisfactory colony for isolation purposes.

c. From two or more organisms of closely related strains—the most undesirable type of colony for isolation purposes.

d. Or from two or more organisms of unrelated strains—an undesirable type of colony for isolation purposes. This type or a contaminated pure colony is sometimes useful in procuring a new proportional mixture of strains.

Broadly speaking a colony may consist of any number of organisms from one to infinity. Technically speaking a colony consists of the organisms confined within a certain radius inside of or on the surface of the mass of colloid gel. For purposes of discussion, let us define a bacterial colony as the uncontaminated descendants of a very small number of organisms, irrespective of the medium in which they are found. It will be realized that this definition covers perfectly the biological factors involved in the derivation of any ordinary agar or gelatin colony.

Colony methods available for the isolation of anaerobes are the following:

I. Agar colonies—von Hibler and older workers used also gelatin.
  A. Surface colonies.
    1. On plates.
    2. On tube slants.
  B. Deep colonies.
    1. In Petri dishes.
    2. In deep agar tubes.

II. Colonies in liquid media.
  A. Isolation of a single bacillus by the India-ink method.
  B. Isolation of a single bacillus or of a small number of organisms by the technique of Barber.
  C. Isolation of a single bacillus by the technique of Schouten, of Hecker, of Holker, or that of Malone.

Isolation from surface colonies has been employed by many workers with anaerobes. Veillon and Zuber list a large number of types of anaerobic apparatus, von Hibler (1908) gives a bibliography of various plates and apparatus for purposes of anaerobic culture, Besson's textbook figures a number of arrangements, and Fildes describes various methods at the end of McIntosh's report.

Henry uses plates of agar which he streaks with egg-albumen and incubates in hydrogen. Stoddard uses slants made of the modified egg medium of Stitt, made with tryptic broth and 1 per cent glucose. Zeissler, who at first used glucose agar plates containing human blood for the isolation of anaerobes, later employed horse blood and sheep blood agar plates. Many laboratories now make use of large slants of blood agar, kept under anaerobic conditions for the isolation of anaerobes. Isolation of nitrogen fixing organisms was accomplished by Winogradsky by inoculating cultures on pieces of carrot which he placed in vacuo, and Fribes isolated pectin fermenters on potato slants rubbed with chalk. McIntosh prefers agar slants to plates for isolation procedure. He reiterates: "It cannot here be impressed too strongly on the worker that the purity of a culture can only be tested and controlled by repeated surface cultivation," and he speaks of the Veillon-tube method of continental workers as giving impure cultures. I have used plating occasionally and am familiar with technique necessary to make anaerobes grow on plates. In fact it was the first method for the isolation of anaerobes that I learned to use. It is a perfectly feasible method, but I find it to be less satisfactory than others for various reasons.

The difficulty of regulating the amount of moisture on the surface of the plate or slant is the primary drawback to the use of surface methods. Aerobic cultures differ fundamentally from anaerobic ones in this respect. They are, so to speak, self-regulating in their moisture content. When a plate is poured, the surface of the agar is exceedingly moist, and the organisms planted in it grow rapidly till their growth is inhibited by the drying atmosphere of the incubator. Moisture conditions are fairly uniform in ordinary bacteriological technique; colonies of most species are discrete and characteristic. The colonies, when few, are usually pure; the viable aerobes usually all form colonies, and the method as a whole is easy and practical. But with anaerobes the moisture content of the medium and the moisture on its surface become of great importance. I have known agar in deep tubes of medium, which had given perfect results with blackleg colonies, to refuse to give a growth of blackleg when it was somewhat old and dried out, though the agar had nowhere, as yet, separated from the side of the tube. The addition of sterile distilled water made the medium as fertile a soil as fresh agar. I have encountered aerobes which grow to the surface of the agar of a shake, but not in colonies upon its surface. How much more would dryness affect the growth of the more delicate anaerobic organisms on the surface of a plate! Even the hardy tetanus organisms, which grow well in dry deep agar, often refuse to grow on its surface. In order to produce discrete anaerobic colonies plates must be dried after pouring. They must be dried just long enough and not too long. This period varies with the composition, age and thickness of the agar, with the humidity of the atmosphere, and with the moisture present in the anaerobic jar. It takes time and patience to learn to adjust the period for drying the plates. Then when the culture is sown and the plates are ready to incubate, what have we for anaerobic methods? A variety of available atmospheres for the growth of the organisms almost as great as is the number of workers in the anaerobic field: Hydrogen, carbon-dioxide, nitrogen, illuminating gas, nitrogen-hydrogen-carbon-dioxide and vacuum with varying degrees of moisture, pressure and oxygen present. How can one hope to standardize type colonies under such conditions? And what, may we ask, *is* the proper moisture for the surface of a plate? There is no universal proper moisture. Agar moist enough to grow tetanus will allow the spread of *B. sporogenes* till the *B. sporogenes* has increased a million times more than the tetanus has. Some mixtures of organisms allow isolation of their components by surface methods, and some do not. When discouraged with plates that have dried too long, the worker dries them less, and finds to his joy beautiful discrete colonies, some round and some lobed. He must fish them immediately onto plates or into a deep medium or they may die. But let him beware of a pitfall. Let him hold them to the light without a cover and look between the colonies. A slight film of moisture there may represent a spread of growth which contaminates all his colonies. But such a spread may be difficult or impossible of detection. A fragment of coverslip dropped between colonies may show bacilli. I venture to suggest that it is almost impossible to determine in an agar slant the non-existence of such a thin spread, and such a thin spreading film is far more likely to occur in the confines of a tube than on a plate.

Methods of spreading a culture on a surface do not separate the individual organisms from one another so well as does a shaking in liquid agar—in properly made shakes the colonies are beautifully distributed.

Other minor disadvantages of a surface method are that the plates must be incubated immediately after sowing and be fished immediately after opening; they are usually valueless when reincubated after opening for inspection because of too much drying, and they require the use of more glassware than do deep-tube methods, and also the use of an anaerobic jar or other anaerobic apparatus.

The method of Marino should be recommended for organisms which form minute colonies, and for demonstration plates. Marino poured inoculated agar in the upper half of a Petri dish, and covered it directly with the inverted lower half, and covered the whole with a larger Petri dish. This method is convenient but not necessary for photographic work, as sections for that purpose may be cut from tubes of agar and may then be mounted between cover and slide. Fehrs and Sachs-Mücke used a similar method, covering the agar with a photographic plate. Krumwiede and Pratt used Marino's method satisfactorily for the isolation of fusiform bacilli, sealing the open crack with wax. Rhein used it with satisfaction for general anaerobic work, pouring a sterile agar layer on either side of the inoculated one. Dick used the method of Rhein, replacing the top dish by a layer of paraffin. All these methods are probably preferable to surface plating for isolation purposes, but are somewhat cumbersome.

Foth complains that the invention of new anaerobic methods has become a sort of sport. Many procedures are too complicated to use, though most methods will serve well for the cultivation of anaerobes. It would seem as though any method employing sticky black pyrogallic acid and alkali should be avoided, or at least only chosen in the modification of Lentz.

Certain workers with surface methods have charged that deep colony procedures do not give pure cultures. Either type of procedure will give pure cultures in the hands of the critical worker and impure ones in the hands of the uncritical one. But I have found in making a large collection of anaerobes that the cultures from laboratories whose isolation procedure was a deep colony method were more often pure than those from laboratories where surface methods were preferred, and I believe that, with the same amount of labor, the same expenditure of time and material, and the same degree of critique, the deep-colony methods are more successful than are surface ones.

Deep-colony methods have been described by the Hesses, by Liborius, and by Veillon and Zuber, and they have been used extensively by von Hibler, Burri, and by French workers. Von Hibler (1908) preferred deep colony isolation to plate methods because of the fact that water of condensation was likely to render plates worthless.

The selection of a suitable medium for deep-colony isolation is an essential to its success. For general work the primary requirement is that the nutriment in the medium allow every anaerobe present to grow and form a colony. Otherwise colonies may be fished through agar that contains living invisible organisms of other species, and the most deceptive sort of contamination will take place. The medium should be clear and transparent. Our standard agar medium for routine work is made of beef liver. The usual proportion of one part of meat to two of water gave too active a growth and too much gas. The medium is made as follows:

One part of ground beef liver and four parts of distilled water are infused over night, boiled, and strained. To the broth add 1.5 per cent peptone, 0.5 per cent salt, and for ordinary purposes make up with 2 per cent agar pH 7.2 (faintly alkaline to litmus).

When unusually active gas-producers are present, high dilutions and short (twelve hours) incubation periods are resorted to. Such methods always suffice when rapidly growing species are the ones to be isolated. But when slowly growing species are sought in the presence of actively growing ones, other methods are available. To absorb hydrogen, 1 per cent potassium nitrate may be added to the agar (Veillon and Mazé). To prevent the colonies of the rapidly growing types from outrunning the others, use 3 per cent agar or old agar that has partially dried out, or pay particular attention to enrichment of the desired species in the inoculum and employ abundant dilution tubes. Do not depend upon any colony method for the isolation of badly contaminated slowly growing tissue invaders, but resort directly to guinea-pig inoculation. For slowly growing non-pathogenic organisms mixed with rankly growing gas-producers, try a sugar-free agar (von Hibler, 1908).

There is an essential point in the employment of deep colony tubes which must be observed. Otherwise the method is of no more use than any other. Actively growing anaerobes frequently leave their colonies and grow in the agar as though it were a broth.

This happens more readily with some types of organisms than with others. *B. Welchii* is the chief offender and should be avoided by heating whenever possible. A tube in which this phenomenon has occurred is readily identified by holding it to the light with a control. Such tubes are to be regarded as "enrichment cultures." Thus their colonies may be of great use when directly inoculated onto another agar series. They are of no use when inoculated into a liquid medium. The close observation of this phenomenon of "permeating growth" cannot be too earnestly insisted upon.

The deep colonies of anaerobes are highly characteristic. Surface colonies are quite characteristic but are obviously subject to many more outside influences than are deep ones. Often colonies of different strains in the same species are different and sometimes colonies of one type of anaerobe resemble those of an entirely different type. But carefully made agar shakes often give a beautiful picture of the flora of a wound or of a culture. They are very easily observed with a hand lens and may be as closely approached as may surface colonies. Aerobic growth is easily distinguished from anaerobic growth. My routine method of testing for impurity of culture has been to make three dilution shakes on liver agar. The first and second tubes tell whether or not the culture is pure. The third usually furnishes colonies suitable for fishing. I was able to isolate, in two series of three agar tubes each, a strain of *oedematiens* type that had been overgrown 1:500 by a vibrion septique.

Technique of sowing and fishing. Boil the tubes of agar for a minute or two, remove them from the water, shake them, boil them a little longer, shake them again to remove the air, then cool them to 45°. Do not boil them for ten or fifteen minutes or the cotton will become saturated with moisture. For ordinary purposes use three tubes to each culture. For new and important material of doubtful nature or for shyly growing organisms among rankly growing ones, use more tubes. Inoculate tube 1 with one loopful of culture and roll it, tip it, and roll it four or five times. Take a Pasteur pipette[1] of large bore, flame it, draw up agar of tube

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[1] It is to be noted that few laboratory workers today understand the making of strong and serviceable Pasteur pipettes, and I hope to be pardoned for describing so simple an operation. Meeker burners are best for this purpose. Heat the glass in the portion of the flame where the heat is nearly uniform for a considerable distance. In a blowpipe or Bunsen flame this is above the cone; in the flame of the Meeker burner it is half an inch above the base. Turn the glass

1, expel it, draw up fresh agar and expel it into tube 2. For cultures containing abundant organisms, give tube 2 2 inches of agar measured in the capillary portion of the tube. For ordinary cultures give 5 inches, for *B. Novyi*, etc., give about two capillaries full. Place the inoculum throughout the length of the agar while withdrawing the pipette, but do not blow air into the agar of tube 2. Roll tube 2. Flame the Pasteur pipette. By means of it place agar from tube 2 in tube 3 to the amount of 0.5 to 1 inch on the upper or thick portion of the Pasteur pipette. Roll the tube. Incubate aerobically at 37°. If actively growing species are present, incubate twelve hours. Otherwise incubate eighteen to twenty-four hours. For blackleg, clostridia, and unknown shy types, incubate four days. Examine the colonies with a hand lens. Look for permeating growth. It is better, in fishing from a tube containing more than one type of colony, to fish once more onto a series of agar tubes. Final isolation should be made from colonies of mixed cultures. Study the tubes carefully with a hand lens, noting minute colonies and aerobic growth. Select the tube to be fished, and, if possible, select the colonies desired. Take a well-made, strong Pasteur pipette of fairly large bore, bend it at right angles where the capillary begins, break the tip, flame the whole capillary. Remove the plug from the tube and loose fibers of cotton from its opening, insert the Pasteur pipette along the side to the bottom, remove and empty it of agar; re-insert it, and blow the whole column of agar into a sterile Petri dish. The large Pasteur pipette may be used many times. One-half Petri dish serves for each tube. Take a short-stemmed Pasteur pipette, hold it in the flame, draw the capillary out to a hair-like tube, and break it off fairly short. Suck up the desired colony and expel it into a tube of meat medium or tube 1 of another agar series. Draw out the

constantly but slowly in the *same* direction, *not* forwards and backwards. Continue till the hot portion softens and contracts to about four-fifths of its former diameter. Never pull the glass while it is in the flame. Remove the rod from the flame and *wait* a second, then pull *slowly*. If the glass is pulled too soon or too quickly the fine bore is formed from the hottest portion only, and not from all the heated glass, the bore is small, and its walls are thin and weak. An hour's continuous practice is necessary to begin with; the art, once learned, is extremely useful and is not forgotten.

pipette again, flaming it well, and use it to isolate two or three more colonies. Other workers employ other methods, which are probably as good. Burri (1902) recommends the use of tubes open at both ends with an autoclaved rubber stopper placed in the lower end. Some use the loop only for purposes of dilution. Some heat the end of the test tube and expel the agar column by force of the steam thus generated. It is necessary to break the tube at the bottom only when an aerobe is present. Burke used a dissecting lens with stand for fishing colonies; Dr. Meyer finds a binocular a great help in some cases. He sections the agar with a sterile blade when researching for minute colonies that are rare. Some workers prefer to attach a rubber tube or a teat to the pipette used in fishing. It is theoretically wrong to fish the colonies from the top of the column of agar without removing it from the tube, because the capillary may pass ungerminated organisms, but such a method might prove practical when used with discretion. Some workers fish the colonies with a platinum needle, but this would hardly prove as satisfactory as a pipette method.

Methods of single-bacillus isolation. Isolation of a single bacillus has been resorted to for the separation of anaerobes. Miss Robertson found that the India-ink method of Burri (Besson 1913) exposed the organisms too much and they failed to germinate. I used the Barber method for some time for blackleg and vibrion-septique organisms, and found that the exposure killed vegetative forms and that spores were necessary to give a growth. I fished from apparently pure cultures various numbers of organisms, from one to ten, into meat tubes and used for a type strain the tube that grew and had received the fewest bacilli. I found the method wasteful of time, material, eyesight, and nervous energy, and have abandoned it. My employment of the apparatus was, however, far from being as skillful as that of Dr. Barber. I explained my difficulties to Dr. Barber and he (1920) has made a careful statistical study of the behavior of various anaerobes when isolated by his technique. He was successful when inoculating various media with different anaerobes in securing 62 growths from 400 single bacilli, and 93 growths from 211 single spores. Vegetative rods of vibrion-septique were particularly sensitive to the air. Barber found the semi-solid medium of Lignières excellent for securing growth of single anaerobes. Colonies could be secured from spores in a chamber containing a *Pseudomonas pyocyanea* culture.

Malone and Holker have devised pipette methods for single bacillus isolation with which I have no personal experience. It may be that they protect the organisms from air better than does the Barber method. Hecker makes interesting technical suggestions. Hort objects to all methods of single bacillus isolation from liquids and he objects to capillary methods on account of optical difficulties. The method preferred by Hort, the perforated plate method, is too aerobic for our purposes.

IX. *Animal inoculation* is frequently resorted to for the isolation of anaerobes. The guinea-pig is the best animal for this purpose. It is highly susceptible to infections and also develops very characteristic lesions, of diagnostic value. Animal inoculation is of prime value for recovering pathogens that have been badly overgrown. It is the only way I know of to recover blackleg organisms that have been grossly contaminated. A drop of lactic acid may be used for a second trial, if the first fails. Inoculation of mixtures from the involved tissues of gas-gangrene cases is of course necessary, but it is likely to give misleading ideas as to the flora of those tissues. It is advisable to run deep-colony cultures from various portions of an amputated limb or of material derived from tissue-puncture or from a blood culture, and to inspect the flora of the limb carefully. A culture or smear from the wound itself may give very misleading data as to the etiology of a gangrenous process. I believe that the rôle played by *B. Welchii* in gas-gangrene nfections has been grossly exaggerated because of the failure of many workers to study carefully the flora from various portions of infected muscle. When the colony method has given pure or apparently pure cultures, inoculate them into guinea-pigs and record results. When immediate identification of pathogens is urgent, examine smears made by puncture from affected tissue *remote* from the wound. Conjecture the possible types there represented, inoculate a series of guinea-pigs with antitoxic or antibacterial sera of the groups probably represented on the smears, in such a manner that for each type of organism there is a guinea-pig immunized against the other types only. Then inoculate the mixed material into all the guinea-pigs. This method was found successful by the Committee. In large war hospitals collections of guinea-pigs immunized by bacterial inoculation have been kept for diagnostic purposes.

It is best to inoculate guinea-pigs in the thigh muscles. Take cultures from various points in the body. The heart-blood culture is usually the most valuable. *Oedematiens*-group organisms and some other pathogens do not always become septicemic, however. Bifermentans-group organisms and other proteolytic types may become septicemic. Inoculate into another guinea-pig a culture from the heart-blood in smaller quantity than was used before. If this fails, isolate the proteolytic organism, immunize a guinea-pig with it, then inoculate the mixture. For all animal work keep a careful record of the cultures inoculated, incubation periods, lesions in the animals, and, above all, make constant use of the microscope.

Anaerobic organisms should be sought in the following pathological conditions:

Infected wounds (rods or cocci).
Gangrene.
Oedema.
Emphysema of muscles, connective-tissue, liver or other organs.
Haemorrhagic condition of muscles.
Pneumonic processes where anaerobic infection is suspected, pulmonary gangrene.
Necrosis of muscle or connective tissue (*B. necrophorus et alii*).
Injection of serous surfaces, especially in ruminants.
Abortion in animals (search foetus for *Bact. abortum*).
Endometritis, post abortum or post partum (*Streptococcus*).
Appendicitis and various ulcerative and suppurative conditions.
Tetanus (in absence of wounds and uterine infection, search for peridental infection).
Botulism, intestinal content and wall, liver, spleen, stools from patients.

Obscure fevers, measles, scarlet fever. Blood cultures, look for various invaders.
Rhinitis, Vincent's angina; mucous surfaces.

Make smears of affected tissue, make meat or brain cultures and make at the same time numerous shakes in deep liver-agar. Examine shakes twelve hours after incubation if possible, and examine meat cultures twenty to twenty-four hours after incubation. Blood cultures in broth, meat or agar should always be made, if possible, *ante mortem* and *post mortem.*

I wish to express my thanks to Dr. Karl F. Meyer for his active interest and coöperation in this work.

SUMMARY

As an aid to the isolation of anaerobes the following notes may be observed:

1. Success in the isolation of anaerobes depends more on the critical sense of the worker than on the method he employs.

2. Microscopic observation should be made of incoming material and of cultures after twenty-four and forty-eight hours' incubation, and the development of a critical eye for the morphology and staining reactions of anaerobes is imperative.

3. Heating of material should be executed according to the logical requirements for that material. Heating at 70° in pipettes is to be recommended for routine work.

4. A routine medium should be employed which will favor as many diverse forms as possible. Chopped beef heart, preferably containing a little peptic digest broth, the reaction at about pH 7.2, presents numerous advantages as a routine medium, for most of the anaerobes studied in a pathological laboratory. If freshly boiled it is usually quite unnecessary to incubate it anaerobically.

5. Selective media may be employed for special purposes, and they offer many possibilities.

6. Isolation by means of guinea-pig inoculation, securing the organism from the heart's blood or from the affected tissues remote from the site of inoculation, is preferable for invading patho-

gens, but may not be depended upon to give a true picture of the pathogenic flora of the material injected.

7. The making of dilution shakes in deep agar (method of Liborius and of Veillon) is to be preferred to other colony methods; care must be taken to isolate for a type a colony from an apparently pure culture.

8. A medium for dilution shakes should afford an opportunity for growth to just as many species as possible. Such a medium is pepton-liver agar, as described in the text.

9. When once pure, a culture should be carefully kept pure. Re-incubation, prolonged incubation in closed jars, storing in closed cans or in dusty places, are to be avoided. Autoclaved media only should be employed for the preservation of type cultures; one cannot be too careful as to routine technique.

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XVII.

SUGGESTIONS CONCERNING A RATIONAL BASIS FOR THE CLASSIFICATION OF THE ANAEROBIC BACTERIA[1]

STUDIES IN PATHOGENIC ANAEROBES. IV

I. PRELIMINARY PAPER

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During the past three or four years I have made a study of a carefully controlled series of certain groups of anaerobic strains secured from pathological material. Attention was directed almost entirely to such strains as were found to be capable of penetrating living guinea-pig muscle in doses of 1 cc. or less of young ground beef-heart culture. *B. Welchii* was not considered, and this organism is not included in the following list. Though I was forced for want of time to neglect non-pathogenic forms, such organisms are so frequently encountered in a study of pathological material that one who has collected anaerobes of one group must necessarily observe those of other sorts and learn something of their ways. The pathogenic tissue-invading strains included in my collection are 80 in number: 23 from human wound infections, 32 from cases of so-called "blackleg" of cattle, 10 from cases of braxy and of blackleg of sheep, and 15 from other animals. The collection includes 30 odd strains of tetanus and other proteolytic organisms of various sorts.

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[1] This work and that described in the following papers was commenced during the author's tenure of the Alice Freeman Palmer Fellowship of Wellesley College.

All strains were carefully isolated and the cultures were continually observed in order to detect contaminations.[2]

The samples collected, though they do not exhaust the pathological material from anaerobic infections, are very widely representative, and the collection of much more material and the isolation from it of many more pathogenic strains would be an exceedingly arduous task. It must also be borne in mind that in greatly increasing the number of strains under observation one must necessarily relax the vigilance with which he criticises the purity of those strains which are studied. The examination of any considerable number of anaerobes is a comparatively new task, and any proposal for classification which is made at the present period is bound to be a temporary one. The time is unquestionably not ripe for an elaborate study of several hundred strains of any particular type of anaerobe because the material for such a study has never been collected, and such a collection would represent several years' work and a considerable outlay for experimental animals. But the cultures that I have been able to isolate during the past few years have furnished so much material for investigation, and the information gained from them has so radically altered my attitude toward the anaerobic group, that I feel that the time has arrived to state my results, to organize them as consistently as may be, and to propose a system for their classification. In other words I feel that the status of the classification of the anaerobes is today so chaotic and unsatisfactory that a pioneer effort at a logical grouping according to our present knowledge is very much needed. If we consider the fact that the investigated material, when compared with the vast amount of uninvestigated material, is exceedingly scanty, we shall not expect such a classification to be final. We have today, however, a fairly definite conception of the pathogenic anaerobes, and by analyzing the groups thus studied we shall be enabled so to orient our classification of the whole anaerobic group that some conception of its size and general relationships will be presented for the use of future investigators.

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[2] An account of the affinities of the animal strains studied will be found in part in The Journal of Infectious Diseases for November, 1920, Vol. 27, and in full in the Collected Reprints of the Hooper Foundation for 1921, Vol. VI, under the title "Etiology of Acute Gangrenous Infections of Animals." This paper contains a description of the methods employed in the isolation of my cultures.

The uncertainties prevailing in the classification of the anaerobes, apparent to anyone who has tried to identify an isolated anaerobic strain, become more glaring as one proceeds to study several strains that were handed to him under the same name, but which display great differences in their behavior. Later, on continuous study of anaerobes of various types, and of the literature of anaerobic infections, the worker comes to the realization that identifications by means of descriptions found in the existing literature can at best be only tentative and approximate, and that the majority of those type strains on which were based the descriptions to which we are compelled to refer for priority are now lost or badly contaminated. Thus a large number of the older descriptions are potentially invalidated, or orphaned, so to speak, and cannot, today, be used for any definite systematic purpose, and the names proposed in those descriptions are now *nomina nuda*. This situation must be deliberately faced. What we need is an elastic, adaptable system of classification in which the old descriptions can find a place as well as the new: a system consistent, also, with the Rules of Botanical Nomenclature, whose adoption has been proposed by the Committee of the Society of American Bacteriologists.

FORMER CLASSIFICATIONS

It is necessary to consider what have been the methods of classifying our group that have been proposed by other workers.

Zopf defined the genus *Bacillus* as including: "Cocci and rods with spores" and the genus *Clostridium* as: "Like *Bacillus* but spores in spindle-shaped elements."

Kruse (1896, pp. 67 and 185) included in his family of *Bacillaceae* three groups which comprise the anaerobes. They are:

6. The malignant oedema group: large spore-bearing anaerobic bacilli. Saprophytic or parasitic. Colonies on agar usually stellate.

No change in form of mother cell on spore formation. Less easily stained by Gram method than preceding group (anthrax group). Usually liquefy gelatin and produce foul odors.

7. The symptomatic anthrax and butyric acid group. Large bacilli that swell to form clostridia on sporulation, saprophytic and parasitic, mostly anaerobes.

8. Tetanus group. Fairly large bacilli with drumstick (Knöpfchen) spores. Mostly anaerobic parasites and saprophytes.

Migula (1900) included in his family *Bacteriaceae* the peritrichially flagellate rods in one enormous genus, *Bacillus*; the non-flagellate rods in another, *Bacterium.*

Fischer (1903) in his second classification divided the *Bacillaceae* into: Sporulating and non-sporulating rods, sporangia unchanged in shape, *Bacillieae*; and spore-bearing rods modified in shape: spindle-shaped, *Clostridieae,* and drumstick shaped, *Plectridieae.*

Lehmann and Neumann (1904) divide the *Bacteriaceae* into the genera: "*Bacterium,* without endogenous spores, rods usually under 0.8 to 1μ in diameter," and "*Bacillus,* with endogenous spores, rods often more than 1μ in diameter."

Orla-Jensen (1909) in his comprehensive reorganization of systematic bacteriology according to the chemical behavior of the organisms, proposed for the higher bacteria the order *Peritrichinae,* to include rods and spherical forms which show a marked tendency to split carbohydrates and amino-acids. In this order he proposed four families, two aerobic and two anaerobic, two producing acid and two producing alkali. The anaerobic families he called *Butyribacteriaceae* and *Putribacteriaceae.* He would place with these, I presume, the anaerobic cocci, and spore-formation is evidently not a requisite criterion for admission to the anaerobic groups. In the *Butyribacteriaceae* he proposed three genera: *Butyribacillus* (to include *B. Welchii* and *B. Chauvoei*), *Pectobacillus,* and *Cellulobacillus.* In the *Putribacteriaceae* he proposed the genera *Putribacillus* and *Botulobacillus,* the latter to include *B. botulinus* and *B. tetani* on account of their toxin production.

The Committee of the Society of American Bacteriologists (1920, classification first formulated in 1917) places in the family (no. VII) *Bacillaceae*, sporulating rods, two divisions which are ranked as genera. These are: 1. The genus "*Bacillus* Cohn 1872 Aerobic forms. Mostly saprophytes. Liquefy gelatin. Often occur in long threads and form rhizoid colonies. Form of rod usually not greatly changed at sporulation. The type species is *Bacillus subtilis* Cohn:" and genus 2: "*Clostridium* Prazmowski 1880 Anaerobes or micro-aerophiles. Often parasitic. Rods frequently enlarged at sporulation, producing clostridium or plectridium forms. The type species is *Clostridium butyricum* Prazmowski."

Breed, Conn and Baker (1908) commented at length on the major divisions proposed by the Committee: "This family, for the spore forming rods, has very good justification. The two genera, *Bacillus* and *Clostridium* can probably be separated, but whether on the basis of relation to oxygen or of shape of the sporangium, the future must decide. Although relation to oxygen is a very important physiological distinction, it must be admitted that the selection of a physiological basis for the separation of these two genera is rather unsatisfactory. It places some of the polar-spored organisms in one genus, some in another, and raises the question where to place facultative anaerobes like *B. mycoides* and *B. cereus*." These authors proposed a family (5): "*Bacillaceae*, rods producing endospores, usually Gram-positive. Flagella, when present, peritrichous. Primarily saprophytes secreting proteolytic enzymes. A few parasites." This family would include the majority of the anaerobes and many aerobes.

Buchanan (1918, a and b) reclassified the bacteria, using physiological characters far less than did the Committee. His classification of the non-acid-fast members of the family *Bacteriaceae*, sporogenous rods, is as follows: Tribe 1. *Bacilleae*, endosporogenous rods, with four genera: 1. *Bacillus*, Aerobic rods, usually Gram-positive, as a rule liquefying gelatin, spores usually not distorting rods when formed. 2 and 3. Anaerobic or micro-aerophilic usually: 2. *Plectridium*, spores produced at extreme tip of cells, forming typical drumsticks. 3. *Clostridium*, spores not produced at extreme tip of cells, at least not forming drumsticks. Cells usually somewhat swollen when spores are formed. 4. *Metabacterium*, with usually a number of spores within a swollen cell. Tribe 2. *Bacteriaceae*, not producing endospores.

Rahn (1920) defines the anaerobes as sporulating rods that store up Iogen (granulose) with or without glycogen. He believes that further research would show the possibility of changing any spore-forming anaerobe into another.

THE CHARACTERS USED FOR CLASSIFICATION

Let us consider the value in classification of the characters whose use has been proposed in the above arrangements.

Morphology of the vegetative·cell. Most of the authors define the *Bacillaceae* as rods. Exceptions to this rule are the arrangements of Zopf (1885) who believed in the transformation of bacterial species, and of Jensen (1909), who used physiological characters for his classification. Both Zopf and Jensen state that spherical forms may be included in such a family. Apparently the unity of origin of the cocci has never been settled by systematists. Winslow and Winslow say (1908): "Yet a consideration of the properties of the members of the group makes it clear that they are mutually interrelated and all sharply separated from the rod-shaped bacteria, except perhaps at one end of the series which they form." Breed, Conn, and Baker do not consider the question of the unity of the origin of all spherical bacteria as settled. The series of strains which Winslow and Winslow used for their study did not, I believe, include any strict anaerobes. Many cocci are facultatively anaerobic. Strict anaerobes of this group are only occasionally met with, but it does not follow that their occurrence is very rare, because the usual technique for the isolation of anaerobes involves some heating process that eliminates the non-sporulating organisms. Anaerobic cocci have been described by a number of authors. Ozaki reviewed the subject (1915). There are, in his list, four diplococci, three micrococci, and four staphylococci, one of these latter appearing at times as a streptococcus or as a micrococcus. Anaerobic streptococci are, according to Hüssy and Bondy, normally human saprophytes which may become parasitic. They are commonly found in cases of puerperal endometritis, according to these authors and according to Schottmüller. Adamson isolated anaerobic diplococci from eighteen out of fifty-one wounds. Sternberg has described an anaerobic streptococcus. Anaerobic cocci have been found in the blood of scarlet-fever patients by Dick and Henry and in that of measles patients by Tunnicliff. Beijerinck (1906) finds a sarcina in soil that is a fermenting anaerobe. Winslow and Winslow did not find the arrangement of the cells of the cocci a good basis for classification. About half the anaerobic cocci produce gas in sugar media. Probably nothing can be decided as to the real affinities of these anaerobic cocci until the chemical behavior of some of them has been studied by one worker and has been compared with that of the anaerobic rods. A morphological basis, when one considers the varied types of anaerobic cocci, would certainly lead one to conclude that anaerobiosis had been independently acquired by certain strains of the various types. Here, as in many cases, morphological and chemical criteria flatly contradict each other. But form of cell (sphere, rod, or spiral) is certainly to be recognized as a much more fundamental character than is the arrangement of cells. Nevertheless, Almquist found that bacilli may grow as spheres at low temperatures. Yet it would seem that the anaerobic cocci do resemble other cocci in their chemical behavior more than they do the anaerobic rods (see Adamson, 1918–19, p. 394). So for the present it is advisable to exclude them from an anaerobic group which contains rods. The chemical study of the anaerobic cocci should be more extensive before they can be placed anywhere.

Motility. The possession of flagella was used as a primary character for classification by Migula, spore-formation being given a secondary place. Chester followed Migula in crediting importance to this character; but other workers have not done so. The Committee (1920, p. 516) state: "The prominent place given to motility seems to us to constitute a peculiar infelicity in these schemes." And Winslow and Winslow (1908, p. 52) do not find motility correlated with other characters in the group of the *Coccaceae*. It would seem that for our purposes the character of motility was entirely unsuited for the making of major divisions. Thus organisms in general so similar as *B. Welchii* and *B. Chauvoei* were placed by Migula's system in entirely different groups because one was flagellate and the other was not. And aerobes of many sorts and proteolytic anaerobes dwell in his work peacefully side by side with *B. Chauvoei* in the enormous and unwieldy genus *Bacillus;* while *B. Welchii* on account of its nudity is relegated to the genus *Bacterium* along with *Bacterium tuberculosis* and other strangers.

Spore formation has been turned to by many classifiers as an important character for the subdivision of the rod-like forms. Zopf, Kruse, Fischer, Lehmann and Neumann, the Committee, and Breed, Conn, and Baker have used it as a basis for making their primary division. It is probable that this is a character of far more value than is motility. It is, however, true that, though there are many similarities between the sporulating rods, we have no proof that they are more closely related to each other than they are to some of the non-sporulating rods, or that the formation of spores originated with any one type. Should we accept such a hypothesis, we should still be unable to show that certain sporulating rods had not lost their power of spore-formation. This power is certainly an advantage to a species, and on that account anaerobic forms losing it are not so likely to persist as are others. But that does not mean that such a phenomenon may not occur.

Kruse strenuously protests (1896, p. 81) the use of spore-formation as a primary character. Moreover the adoption of spore-formation as a character for the subdivision of the rods would make us exclude from the anaerobic group such organisms as *B. egens*, *B. necrophorus*, Bacillus D of Adamson, *B. fragilis* of Veillon and Zuber, and probably a goodly number of undescribed organisms which in their behavior closely resemble the sporulating anaerobes. I find that *B. egens* and another Gram-positive pathogen are so similar in many respects to *B. Welchii* that they might easily be identified as one species by many workers, and it is evident that only an artificial classification would separate them, yet *B. egens* and the other pathogens have not been shown to produce spores. *Asporogenous* anaerobic rods have not been frequently reported, but when one remembers that the preliminary step in isolating anaerobes is usually a heating process, it will be clear that the proportion of anaerobes that do not sporulate may be considerably greater than one would estimate on the basis of published descriptions. The soil mixtures from which Weinberg's organisms, *B. egens* of Stoddard, and my above-mentioned pathogen were isolated had all been subjected to a physiological weeding out in human tissue before they were inoculated into media, and a colony method without heating was thus practicable for isolation purposes.

The matter of a primary division of the rod-shaped bacteria then simmers down to a question of whether an anaerobic habit or a spore-forming habit is the more fundamental one. It is perfectly evident that certain asporogenous anaerobes have closer physiological affinities with certain sporulating anaerobes than the latter have with the sporulating aerobes or even with most other anaerobes. We may have in the power of sporulation such a phenomenon as that noted among the insects: there are primitively wingless insects, the *Thysanura*, and there are various types of insects, such as the *Siphonaptera*, and the *Mallophaga*, which have lost their wings, and there are insects that have no wings at the time or in the form that we happen to observe them—larvae and pupae and worker ants and apterous mutants of winged forms. Therefore the possession of wings, conspicuous insect characteristic that it is, has been discarded as a character for the separation of insects from other forms. The whole question is reduced to the much agitated one: Are we going to give precedence to physiological or to morphological characters in the classification of the bacteria?

Morphology of the sporangium has been used as a character by a long succession of workers. Its use may be more vigorously attacked than that of the other characters. It was introduced by strict morphologists (Zopf, Fischer) entirely independently of any physiological criteria, and in this way was quite justifiable because by its use a superficially consistent division could be made. The rods which did not swell at sporulation formed one group, those which did swell formed another. But Kruse and the Committee have superimposed upon this type of classification a physiological one, and the result is a division that it is impossible to carry out. Probably more aerobic rods fail to swell at sporulation than do anaerobic rods, but the exceptions to this rule are so numerous on both sides as to render worse than useless the employment of the morphological character in connection with the physiological one. The reader is referred to the illustrations given by Ford and his co-workers and by von Hibler (1908) and by the Medical Research Committee.

The position of the spore has been used by several authors in subdividing the anaerobes. Species of anaerobes have very characteristic ways of sporulating. But the position of the spore may vary in one species within limits wide enough to render its use exceedingly inadvisable as a character for the grouping of genera. One may take as a single illustration the behavior of a pure strain of vibrion septique. Vegetative forms are fairly uniform on most media. Sporangia, however, show in their variations all the characteristic forms described by Fischer, by the Committee, and by Buchanan. They are, on meat medium (forty hours' culture), usually thickened in the center with the typical form of clostridia. But some rods may contain spores and still have parallel sides, and forms with sub-terminal and terminal spores are nearly always to be found. On serum media the vegetative rods may vary greatly in their proportions, the sporangia assume many fantastic shapes, and "drumstick" forms are common. On the liver of animals the vegetative rods form enormous thick filaments: some strains may sporulate without at all changing their outline or may form clostridia, and some, identical with the first in morphology on ordinary media, may, on the liver of animals, show club-shapes that resemble the clubs formed by the actinomycetes, while others form great globoid masses, terminally or mesially placed in the rods. Rarely three or four spores may occur in a rod which has remained undivided. In fact the only fixed morphological character to be noted is the shape of the spores, which, so far as I know, in this species everywhere remains oval. This is the sole morphological character that was noted in my anaerobic studies that cannot be assailed as inconstant, yet the shape of the spore is a character that has been consistently overlooked by classifying morphologists, who have chosen instead the extremely variable one of spore-position.

The size of the rod has not been mentioned by most classifiers. In general the sporulating rods are larger than the non-sporulating rods. But to reduce this generalization to definite measurements as Lehmann and Neumann have done is not a practical procedure.

The arrangement of the bacilli in chains is not significant. Probably the aerobes form chains more frequently than do the anaerobes, but filament formation cannot logically be used as a character for their differentiation. Winslow and Winslow found chain formation a character of minor value in the classification of the streptococci. Certain organisms, e.g., *B. Novyi*, regularly form filaments on certain media, but the character is of specific, not of generic, value.

Morphology of colonies would be mercilessly discarded by experimental workers as a means of subdividing a large group like that of the *Bacillaceae.* It has more value for lower subdivisions.

The Gram-stain is an impossible character to use in dividing the *Bacillaceae.* It fits neither with the morphological characters nor with the physiological. There are numerous Gram-positive anaerobes and aerobes, and numerous Gram-negative anaerobes and aerobes. (See Heller, 1920.)

Granulose (Iogen) content of the bacterial cells cannot be seriously considered as a general character of anaerobic rods.

Habitat. Winslow and Winslow found that the *Coccaceae* could logically be divided according to habitat. The parasitic forms constituted one group and the saprophytic ones another. Certain anaerobes are frequently inhabitants of the intestines of animals. But this type of character has not been worked out for the anaerobic organisms and should evidently not be used in classification until it has been investigated thoroughly. In view of the fact that a large variety of anaerobes are to be found in soil it is not advisable to state that anaerobes are "often parasitic." Habitat might be used conservatively as a descriptive character.

The formation of toxin and the pathogenicity for animals—the most interesting of characters to the majority of us—cannot logically find a place in the higher divisions of our group. They become of more systematic value in classifying genera and species, but they should always be used in connection with other characters. Jensen's grouping of *B. tetani* and *B. botulinus* in the genus *Botulobacillus* because both produce toxin is not advisable. The toxins produced by these organisms are diametrically opposite in their effect on nerve tissue and that of *B. botulinus* and probably that of *B. tetani* are entirely adventitious so far as a parasitic mode of life is concerned. Other anaerobes of different affinities form toxins that produce still other and different effects.

We have seen with what ease objections may be made to almost any morphological character used for the division of the rods of higher metabolism, in case any physiological character is allowed to enter into the classification. We have stated also that some of the proposed morphological characters are not sound for single species or even for a given culture of a single strain. As Breed, Conn, and Baker say, the future must decide what type of character, physiological or morphological, will predominate in the classification of the bacteria. The two systems are so often contradictory that they can never exist side by side. One must always be used as the chief deciding factor, the other as an auxiliary which may, at any time, give precedence to the former.

Experience with a single group may be misleading. The higher plants, and even the fungi, may be satisfactorily classified on a purely morphological basis. At present the systematics of bacteriology are so tentative that the matter must be left to the judgment, or shall we say to the taste, of those who have themselves worked with the groups that they discuss. I am totally unable to see how morphological criteria can possibly be used to any logical end in the classification of the rods of higher metabolism, or even of the main groups of anaerobes. In 1902 Achalme found morphology of absolutely no use for the differentiation of anaerobes. In 1905 von Hibler energetically decried the use of morphology in anaerobic classification. In analysis of the anaerobic group morphology has its place, and can logically be used to distinguish types that are otherwise similar. It can *not*, in my opinion, be used to unite groups that are otherwise dissimilar. In the anaerobic group morphological criteria alone would hopelessly bewilder the student and lead him to the correlation of fundamentally different types and to the separation of sister rods of the same strain. Morphology need, however, never be entirely discarded from classification. The morphology of the anaerobes is, for a given species, so characteristic, that if it be observed conscientiously, and if the worker does not generalize too freely in formulating his description, it may well be used as a valuable descriptive character for species, and as an auxiliary character for the description of genera. It is in organizing the major groups of anaerobes that morphology fails us. Professor Harvey M. Hall of the Botany Department of this university suggests that after a logical and fundamentally historical chemical classification has been made, morphological characteristics will be found which will be consistent with it. One must distinguish between different types of morphological criteria. Gross form of rod and position of spore are not fundamental morphological characters: they vary greatly within the species. But a highly refined cytological technique such as has never been generally applied to our organisms might reveal consistent morphological characters.

THE ANAEROBIC RODS

In my opinion the most logical division of the bacterial rods—the rods which split higher compounds and are not acid-fast—is the physiological one of susceptibility to free oxygen. Ability to live in the absence of free oxygen has been developed by too many types to make it a character of value. But fewer types have developed a susceptibility to free oxygen. The classification proposed by the Committee (1920) places certain anaerobic forms such as the anaerobic leptotrichia with aerobic forms that are patently their relatives. The Committee justly assigns generic rank to the obligately parasitic, non-sporulating, shyly growing, fusiform anaerobes. They do not mention the anaerobic cocci, which should probably be included with the *Coccaceae*. The Committee does not mention any anaerobic non-sporulating, non-fusiform rods. In my collection there are two such strains (*B. egens* and one of my own isolation) which do not readily attack milk, and these organisms do not fall into any of the groups designated in the key, which follows the Committee's classification. *B. necrophorus*, again, of whose phylogenetic position I am in doubt, does not sporulate.

Fusiform bacilli have probably recently acquired an anaerobic habit through parasitism. Thus Larson and Barron describe a strain of these organisms which became adapted to growth under aerobic conditions. Analogous is the behavior of *B. abortus-bovis*, which frequently refuses to grow aerobically when first isolated but later accustoms itself to living in the presence of oxygen. The anaerobic habit may, in some cases, be due to a sensitiveness to carbon dioxide instead of oxygen. Curtis has described a motile curved anaerobic rod which he isolated from uterine discharges. The phylogenetic position of this organism is in doubt. So also is the position of the branching anaerobes *B. ramosus* and *B. furcosus* of Veillon and Zuber, and the influenza-bacillus-like rod isolated from an abscess by Russ. Tunnicliff reports anaerobic rods from rhinitis and from bronchitis patients; and Tunnicliff, Plotz and his co-workers, and Dick and Henry, report anaerobic organisms in the blood of fever patients. But these organisms grow slowly and do not resemble the chemically active anaerobic rods. We are justified in concluding that an organism which has lived as a saprophyte or parasite in the tissues or in the uterus may owe its anaerobic habit to such residence. There are several reasons why we should hesitate to attribute to a parasitic or intestinal saprophytic history the anaerobic habit of the rods found commonly in soil. These rods are abundant in unmanured soil, their species are very numerous, their metabolic processes exceedingly varied. They may grow under aerobic conditions in company with aerobes and may grow in the presence of oxygen in pasty or solid material or in liquids containing soap or other substances which alter surface tension. But they retain their anaerobic habit on clear liquid or agar media. The commonest intestinal organisms, those of the colon group, have not assumed a sensitiveness to oxygen. Many of the anaerobes, such as those of putrificus, sporogenes, and bifermentans affinities, are the common agents of putrefaction outside the animal body, while others described by Omeliansky are the common cause of the decay of cellulose. When parasitic outside the intestine, these organisms usually show little of the character of true parasites, but cause fulminating fermentative processes which do not pass from affected individuals to healthy ones. *B. abortus*, the anaerobic streptococci, fusiform bacilli, and certain types of *B. coli*, when they invade the tissue may establish chronic infections, characteristic of highly developed parasites, but the anaerobic rods common in soil do not, so far as we know, behave in this manner. They are apparently unable to establish themselves as chronic parasites in tissues which are well vascularized. Had they a history of intestinal saprophytism, we should probably find highly adapted parasites among them, and should find it easy to educate them to an aerobic habit.

No one has, of course, suggested that the nitrogen-fixing anaerobes described by Winogradsky developed their anaerobic habit through parasitism. These organisms are active splitters of carbohydrates. They are usually regarded as primitive. It is more probable that the anaerobes of higher metabolism had an evolution of the following type rather than one from the sporulating aerobes or from intestinal saprophytes of large animals which appeared at a comparatively late geological period.

- Nitrogen-fixing anaerobes that split carbohydrates. E.g. *Clostridium Pastorianum* Winogradsky, a large anaerobic rod that forms oval spores.
- Carbohydrate-splitting anaerobes that can utilize fixed nitrogen but not free nitrogen. They do not produce gross proteolysis. E.g. *vibrion septique.*
- Anaerobes that split proteins very actively. Some but not all have lost the power of splitting carbohydrates. E.g. the sporogenes type.

Geologically this sequence would be the most natural. But we know so little about bacteria and their evolution that any evolutionary arrangement is little more than guess-work at the present time.

It is my intention to propose a division which seems more logical than "the *Bacillaceae,* spore-bearing rods," as distinguished from the "*Bacteriaceae,* non-sporulating rods of higher metabolism." This division implies the creation of a family: "The *Clostridiaceae,* rod-like forms, not spiral, which will not grow within seven millimeters of the surface of a shaft of clear tissue-free agar medium contained in a tube 12 millimeters or more in diameter, incubated in air, in which they are able to grow in the depths. They may or may not possess peritrichial flagella; they may or may not form spores. Most members of the group are characterized by their energetic action on proteins or on carbohydrates or on both of these types of substances." It would be unwise to claim that we have evidence to show that these organisms are descended from a single type—in other words that this is a perfectly logical classification. Bacteriologists have no characters available for purposes of classification whose nature is sufficiently understood to grant us the liberty to make such assumptions. But I believe that this primary division will separate fewer types that are physiologically alike than any other thus far proposed. The energetic action of the anaerobic non-fusiform rods upon carbohydrates and proteins is characteristic and separates them from most other groups. In the present state of our knowledge it is only the separation of types that have several characters in common that is carefully to be avoided. The bacterial characters understood by us are so elementary that we can, as yet, have no assurance that we are not at times uniting types that have not the same ancestry.

It will be noted that the Committee has arranged the bacteria into orders, families, and genera. Most families have also been divided into tribes. The genera of the *Actinomycetales* have not thus been arranged in tribes, because their relationships are avowedly obscure. But the sporulating rods have been given the very inferior position of two genera and the tribal relationships are not expressed. This is because these organisms have been so slightly studied. As Ford says in his introduction, our knowledge of the spore-bearing bacteria is still in a state of chaos. The sporulating organisms, at least the anaerobes, are legion in species, and form a group that is to be divided and subdivided.

Whether or not the aerobic spore-bearers (genus *Bacillus* of the Committee) form a homologous family, I am unable to say. Compared with the anaerobic rods they are apparently very few in number of species. Ford and his co-workers list twenty-eight species which they place, on the basis of morphological and gross cultural characters in nine "groups." These groups would probably form as logical genera as some of the others which have been recognized.

It may be asked why tribal rank should not be assigned to the anaerobic and aerobic rods instead of family rank. It would seem that the group of anaerobic rods is sufficiently large, peculiar, and important, to warrant its being given family rank. Probably none of the botanical or zoological families contain nearly as many species as may be found among the anaerobic rods. On strict analogy with botanical and zoological classifications the anaerobes should command an order at least, but being unfortunately dogmatically confined in our classification to a single class which must include all one-celled cellulose- and chlorophyll-free plants that divide by simple fission, we must be modest in our demands.

In order to ensure a natural classification, characters must be worked out for each group, characters that will to some extent correspond, and show by such correspondence or by the lack of it where lie the historical divisions and where the parallel developments that have taken place independently. This alone is a great labor. For the group of the anaerobic rods and for many other groups nothing of the sort has as yet been seriously attempted. The Winslows' classification of the *Coccaceae*, a pioneer work in this direction, has applied several principles, which may well be heeded in making future classifications. These authors applied to 500 strains of cocci from various sources the biometric principles in use by students of heredity, by anthropologists, and to some extent by botanists and zoologists. Upon a study of the tabulated figures based on the behavior of these organisms they formulated their determination of what to call a species and of how to group species into genera. They found, in common with botanists and zoologists, that when abundant material is at hand it is quite impossible to define as a species one single type. If our methods were sufficiently refined we could probably distinguish every bacterial strain from every other, just as we can distinguish every human being from every other. A species is finally to be determined by comparing the characters of aggregates of individuals (higher plants and animals) or of strains (bacteria), and by selecting the types which occur most frequently as the standard upon which to base specific descriptions. The conclusions arrived at by Winslow and Winslow as to analysis of their data are as follows:

> First, each center of numerical frequency, marking a group of organisms varying about a distinct type in regard to a single definite property, may be recognised as a species. Second, those species which are bound together by the possession of a number of similar properties may be constituted as genera, and larger groups of genera, still characterized by some characters in common, may receive the rank of families or subfamilies.

This method of working is evidently very different from the old method whereby one man described one strain and another man another, and a third decided some ten years later from their descriptions whether they were working with the same or with different species. The biometric method is evidently true biology, while the other is a process of cataloging. The principles of the biometric method are those that one would choose to follow, even though one were unable to make a study of so extensive a series as did Winslow and Winslow. But it is upon the first method, that of collation of descriptions from the literature, that our comprehensive classifications have so far been made. This has led to a complete misunderstanding of the nature of the anaerobic group. A *few* anaerobes have been described, most of the descriptions being wholly inadequate for purposes of specific determination. This fact has in no way deterred workers from making identifications. Some of these mistaken identifications are now thoroughly ingrained in the literature, for example the use of the name *putrificus* in Germany for the *sporogenes* type of organism, when there is a different definite type existent which corresponds far more closely to Bienstock's description of *B. putrificus.* The names of some of the described anaerobes have been accepted, and if these types are pathogenic or very common they find their way into the textbooks. Textbooks mention usually five anaerobic organisms: *B. tetani, B. botulinus, B. oedematis-maligni, B. Welchii,* and *B. Chauvoei.* So far as I can see the classifications are largely based on a conception of the anaerobic world which knows few forms but these. But the worker with "wild" material can easily pick up and isolate two or three new species of anaerobes a day for an almost indefinite period. Few workers now pay any attention to non-pathogenic anaerobes, knowing that their path would be crossed by so many new species that no end but the mere description of new species would be attained. But these undescribed forms are just as important, theoretically, to the systematist, as are the pathogenic ones.

The ideal way of classifying anaerobes would be a biometric one carried out on a scientifically adequate number of strains. But it will be years before sufficient interest in the anaerobes exists to warrant the collection of any such material. The labor of making such collections and of keeping close watch on all strains to insure their purity is tremendous. The bacteriologist is not the only systematist who has to do with such a problem as ours. The classifier of the *Coccaceae* is in the position of the curator of a museum who has before him the skins of a hundred or two of squirrels or other rodents, their measurements and habitat given, their skulls freed of muscle reposing in tiny bottles by their sides. The classifier of the anaerobes is today in the position of the exploring zoologist who sets his traps at night on his journey and catches one or two or three new rats or mice that do not resemble any thus far met by him. Both men describe new species and both serve science in so doing. But the museum worker may use as his type-species the animal whose characters are an average of those of all the rest, while the exploring zoologist must call the "type" one of his chance catches which may be a freak in one or more ways. And yet we would not have the explorer place his mice from a far country nameless in a museum for a future zoologist to describe some seventy-five years hence when the far country has been settled and the mice have been caught by the hundred.

The problems presented in the classification of the *Coccaceae* and of the *Clostridiaceae* are quite different in other ways. The anaerobes form a group of far more diverse types of organisms, both from the morphological and from the physiological standpoint, than do the cocci. One may say that their characters are more salient, more easily perceived, or more definite in their nature, than are those of the cocci. Or one may state with equal truth that the anaerobic group is a less homogeneous one than that of the cocci. One would also be justified in stating that the anaerobic species and genera are far more numerous than are those of the *Coccaceae*. Therefore a representative and adequate collection of anaerobic strains for statistical study would have to contain not hundreds but thousands of strains. But this element of distinctive characters places in our hands a means for the determination of genera before we are familiar with many strains of each genus.

SPECIFIC AND GENERIC CHARACTERS

No one will dispute that the decision as to the line between specific characters and generic characters lies with the systematist who, though he have a previous knowledge of many other groups, has confined his attention to one group, and not with the systematist who organizes the published work of others. In different groups these boundaries vary somewhat. But in general the following definition will probably be accepted for such types of material as we are *unable* to examine thoroughly by a biometric method. *Strains of bacteria that regularly and consistently differ from each other in certain characters that we have come to recognize as significant may be assigned to different species. These characters may be quantitative in their nature. It lies in the hands of each worker to decide what the value of these characters is.* An arrangement made without reference to biometric data is in any case bound to be tentative. *Generic characters are based on qualitative properties.* As a working system the following classification of specific and generic characters for organisms of the anaerobic group is suggested:

Generic characters, qualitative:
  Qualitative chemical action: behavior on usual laboratory media (excepting the fermentation of milk).
  Staining reaction and general morphology of individuals.
  General habits of colony formation.
  Pathogenic action.
Specific characters, quantitative:
  Quantitative chemical action: behavior on carefully standardized media; hydrogen-ion end point attained as a result of specific enzyme action.
  Sugar fermentations if not subject to active mutation.
  Peculiar habits of the morphology of individuals.
  Exact behavior of colony formation on a standard medium.
  Details as to pathogenic action.

In studying my material, I find abundant justification for the application of properties of this sort. With such characters to build upon, a more detailed structure, more exactly expressing relationships, can later be erected. We are not yet ready to declare where the limits of variation for the organisms of our group may lie. But I am decidedly of the opinion that in general the old conception of species as accepted for the anaerobic group must in future be taken as the conception of genera, and that we must be more exact in our examination and analysis of these organisms. To do otherwise, and classify as the same species organisms which regularly and consistently show marked quantitative differences in their behavior, would be to distinguish our system of classification sharply from those of the botanists and zoologists and to set up our own meanings of "species" and "genus." The application and use of the characters here suggested will be described more fully in a future paper.

Perhaps the first-noted definite "character," splitting up a so-called species into a number of groups, is the agglutination reaction. Thus Tulloch by this method demonstrated the existence of four types of the group recognized under the name of *B. tetani*. Robertson thus subdivided her vibrion septique strains into four groups, and Henry divided the species of *B. sporogenes* into two groups on the basis of the agglutination reaction. It has been found by various workers that agglutinating anti-sera formed against various strains of *B. Welchii* do not agglutinate heterologous strains of the same organism, though Werner found a serum that agglutinated one out of several heterologous strains. When one thinks, however, of the comparatively numerous cases of cross agglutination recognized in other groups, some of which may, and some of which may not be modified in their importance by absorption-of-agglutinin tests, and when one considers the Weil-Felix reaction, one is ready to look for a more highly specific character than that of the agglutination reaction by which to analyze his strains. The Medical Research Committee term the agglutination reaction "ultra specific" (1919). I personally regard this reaction as of sub-generic rank, and not as of sub-specific rank in the anaerobic group. The agglutination reaction has not yet been investigated thoroughly enough to determine its value as a systematic character for anaerobic bacteria.

NOMENCLATURE OF LOWER GROUPS

Our next concern relates to the nomenclature of our genera and species. We are peculiarly hampered in bacteriological work, when we try to base our names for organisms upon their behavior or characters. Morphology is a notoriously bad bacterial character for generic names, though it has long been used as a generic character for our primary divisions of the bacteria (*Coccus*, *Bacillus*, *Spirillum*) and apparently has a sound basis in this case. Pathogenic action is an equally misleading character upon which to base generic names: most anaerobes are not pathogenic and of those that are, various groups produce gas, oedema, haemorrhagia, etc. Chemical action would be the best type of character for descriptive purposes. But how often might we not, in a group that is so enormous as that of the bacteria, inappropriately name a new genus for a chemical character that was possessed in a greater degree by other genera, or was not possessed by all the members of the genus? There is also a prejudice among botanists against the formation of generic names from specific names, though such forms are not unusual in zoological nomenclature.

Dr. Karl F. Meyer has suggested to me the use, for purposes of generic nomenclature, of patronymics, preferably of the name of the author first describing the original species of a genus. This seems to me the most fitting and logical procedure. It has ample precedent in botanical nomenclature, and has been used in bacteriological nomenclature for years: e.g., *Pasteurella*, *Eberthella*.

Recommendation V.e. (International Rules for Botanical Nomenclature, Chap. III, Sec. 3, No. 3) will, if heeded in the formation of generic names, aid greatly in overcoming conservative objections to the new system of classification. This recommendation reads: "To recall, if possible, by the formation or ending of the name, the affinities or the analogies of the genus." Thus in the group of the cocci, *-coccus* has been accepted as the usual termination of the generic appellations; *-bacillus* has never been popular for such formations, probably on account of its length. I had considered the ending *-ella* as used in 1900 by Lignières for *Pasteurella*, and by Buchanan for *Pfeifferella*. But *-ella*, like *-ia*, is a common ending for generic names among the higher plants. Would it not, in view of the existence of this recommendation, be appropriate to terminate the generic names of bacillary forms with the ending *-illus* (from *Bac-illus*)? (Patronymics ending in vowels may drop the final vowel before adding *-illus*.) Perhaps the ending *-erium* may also be found appropriate for names created in subdividing the old genus *Bacterium*. Specific names should be adapted from the original specific name, if such is valid, and new species can, of course, be named according to the will of the author describing them.

TYPE STRAINS

Because of the factor of variation in the habits of cultures, because many species of organisms must be frequently transplanted to keep them alive, and because of the important rôle played by contaminations, the custom, so long accepted by the botanists and zoologists, of preserving in museums type-specimens of newly-described species, has never been popular with bacteriologists. The facts as related to the anaerobic group are as follows: No experienced investigator of anaerobes would care unreservedly to turn over to anyone else his type-strains for general distribution, because of the ease with which they may become contaminated, and because of the difficulty that the ordinary worker has in recognizing contaminations. The rather generally disseminated view of Grassberger and Schattenfroh (see Heller, (1920)) that the characters of anaerobes are highly variable is one to which I cannot subscribe. This view, which has cast a blight on modern German anaerobic studies and caused grave misinterpretations (see Rahn), has also deterred workers from the use of type-strains to make their descriptions definite. The anaerobic bacteria are fairly stable types (when in pure culture) and they have, in common with all other organisms, that degree of variability which permits them to adapt themselves somewhat to changing conditions and they may, occasionally, show mutations as do all living organisms. The problem of their variability is essentially no different from the problem of the variability of other bacteria. Their behavior toward proteins is remarkably constant, while their action on carbohydrates is somewhat variable.

THE SUBDIVISION OF THE CLOSTRIDIACEAE

We have now outlined the status of the anaerobes in bacterial classification, and the position to be held by genera and species. It remains to organize the structure between the generic rank and the family rank. It is here that we have the most need of allowing room or elasticity for the convenience of future systematists whose information will be greater than ours is today. With our present knowledge I do not think that we are entitled to make more than one main subdivision of the *Clostridiaceae*. This division should follow that made by von Hibler in 1899, in 1905, and in 1908. Von Hibler showed that some anaerobes produce more acid than alkali on certain media, while others produce more alkali than acid. On the basis of this observation he classified the fifteen species studied by himself into two groups. He titrated brain cultures and milk cultures against $\frac{N}{100}$ HCl and KOH, and found that on both media the organisms of the first group produced an acid reaction, while on brain medium, which is poor in sugar, the organisms of the second group invariably produced an alkaline reaction, and on milk, though some of them at first produced an acid reaction, they all finally gave an alkaline end point. The production of an alkaline reaction was always associated with peptonization of milk and was usually associated with a blackening of brain medium and with the production of hydrogen sulfide. The organisms that produced and maintained an acid reaction in milk and brain never peptonized casein or blackened the brain particles.

The division thus made by von Hibler has been accepted and followed by Jensen and by various anaerobic workers. The alkali-producing group is termed *proteolytic* or *putrefactive*, the acid-producing group *non-proteolytic* or *saccharolytic*. The organisms studied by von Hibler were all energetic in their reactions. *B. Novyi* and organisms related to it, such as *B. oedematiens*, form hydrogen sulfide in blood media and do not produce much acid in milk. They do not peptonize casein or blacken or putrefy meat. They and the *Bifermentans* type and certain other organisms that I have encountered do not fit so nicely into von Hibler's scheme that we can safely place them in either group without drawing a dogmatic line and measuring their activities accurately. Douglas, Fleming, and Colebrook describe a sporulating anaerobe, *B. cochlearius*, which shows no marked properties that unite it with either of von Hibler's groups. It therefore seems advisable to define conditions under which anaerobes may be tested to determine their affinities with these two groups. Objection may be made to such a separation of the anaerobes on the grounds that when borderline organisms are in question it is but a cataloging process to separate them. Perhaps so, but when a classification is developing as is this one and when so very few borderline forms are known, a cataloging classification is better than none; later a group containing these organisms may be formed if necessary. Moreover the vast majority of anaerobes do fall definitely into one or the other of these two groups and can be placed where they belong on the basis of their behavior on ordinary media.

The requisites governing the selection of a medium for such a purpose are definite. In the first place all anaerobes must grow upon it. Blackleg organisms usually refuse to grow on simple sugar media. In order to give the organisms a good start, and to furnish material for the formation of acid, our medium should contain a little sugar, of the sort available to the greatest possible number of anaerobes: glucose is probably the sugar which best fulfills this requirement. But this sugar should be little in amount, and sufficient protein should be present, so that the acid formed from the sugar may not inhibit the growth of the organisms and prevent them from carrying the reaction back to an alkaline end point if they are capable of so splitting protein that they produce such an end point in the absence of acid.

Probably tissue-containing media best fulfill the above requirements. They need no more glucose than that which they derive from the tissue contained in them. Brain medium as used by von Hibler would be excellent were it made up with sufficient liquid for titration purposes. Von Hibler titrated liquid from his brain medium with litmus against potassium hydrate or hydrochloric acid. Today workers would prefer to use a hydrogen-ion determination, for which a fairly clear liquid is necessary in case the simple colorimetric method is employed. Bromthymol-blue is a suitable indicator for making this separation. At present most laboratories use the beef-heart medium introduced by Robertson and Martin for anaerobic study because it has many technical advantages over brain medium. It should preferably contain about 5 per cent of peptic digest broth and should be made with twice its weight of distilled water and tubed in large tubes in order that enough liquid may be present for hydrogen-ion determinations on several occasions.

Ten days' incubation at 37° will be found quite sufficient in most cases for such a determination as we wish to make. Von Hibler found the reaction decidedly acid or alkaline in brain medium after five days. But to allow time for weakly proteolytic organisms which form acid from glucose, to carry the reaction over to the alkaline side, we should incubate the cultures for twenty days at least. Such organisms do not at first produce gross signs of putrefaction, but their proteolytic tendencies may be tested for by the lead acetate test for hydrogen sulfide. There may be soil anaerobes which do not grow at 37°. Time and temperature for incubation will have to be decided upon for such organisms separately in case they are found.

Von Hibler (1908, p. 88) found that with his pasty brain medium the mode of incubation, aerobic, or anaerobic in hydrogen or in carbon dioxide, made no difference in the reaction. But if we are to use a medium with a considerable amount of liquid on the surface it would probably be unwise, even though anaerobes grow in the medium with the surface open to the air, thus to incubate our organisms for twenty days. An anaerobic method should be employed. The simplest method available to most laboratories is stratification with vaseline. After incubation the culture should always be boiled to expel carbon dioxide.

For pure culture study the exact point of the reaction of the medium at the time of inoculation is not important. It is important only that the reaction should be well within the limits for growth of the organism studied, and not very far from neutrality. A reaction of pH 7.2 (neutral to litmus) has been used in this laboratory for most anaerobe media. The selection of a reaction point for the dividing of the two types of anaerobes is more difficult. Von Hibler (1908, pp. 89, 104) used the neutral point of litmus. The selection of a dividing point in meat medium is frankly an arbitrary affair. I suggest pH 7.0, the neutral point of hydrogen-ion concentration, for the dividing line between the alkali-producing and the acid-producing anaerobes.

It is unlikely that any other division of the *Clostridiaceae* will be made which would be placed above the division into the two groups just described. But I am certain that various arrangements of the genera which compose these groups will in time be made. There are many anaerobes that behave quite alike in one way and entirely differently in other ways. If this were to be a final classification of the group, or if one wished to outline a temporary classification, one would give the division into putrefactive and non-proteolytic anaerobes a tribal rank. But I am quite certain that the makers of future arrangements will want very much to utilize tribal and subtribal ranks for other purposes. I had myself thought of dividing the non-proteolytic anaerobes into two tribes or subtribes on the basis of presence or absence of flagella, but Dr. M. Christiansen has repeatedly been unable to demonstrate flagella on his whale-septicaemia bacillus which so closely resembles the vibrion-septique type of organism that they should probably be placed in the same genus. But tribal and sub-tribal classifications are sure to be made by someone before long and it will be a better provision for the future to give the proteolytic-non-proteolytic division sub-family rank.

I propose, therefore, the two subfamilies, *Clostridioideae* and *Putrificoideae.*

Clostridioidcae: Clostridiaceae which on meat medium produce after twenty days' incubation under oil at 37° a reaction of pH 7.0 or a more acid reaction, the reaction being read after the culture has been boiled.

Putrificoideae: Clostridiaceae which on meat medium produce after twenty days' incubation under oil at 37° a reaction of pH 7.1 or a more alkaline reaction, the reaction being read after the culture has been boiled.

The name *Clostridioideae* is derived from Prazmowski's generic name *Clostridium.* The name *Putrificoideae* is formed from the specific name *putrificus* (Bienstock 1884). (*Putribacillus vulgaris* of Jensen.) We are hard put to it to find sufficient generic names upon which to form appellations for higher groups in the anaerobic field because *Bacillus* was the generic name applied to any and almost every rod described. But I think that bacteriologists will be justified in using ancient specific names for the formation of the names of tribes and families. Such a proceeding would have a basis in logic if not in precedent. The name *Putrificus* has probably been used for various organisms of the same genus (as well as for those of other genera) and is as much a generic name in sense as though it had been originally designated as such. I should, for example, on finding a slender proteolytic rod that formed terminal oval spores and did not split glucose or other sugars, term it *putrificus type,* knowing that more organisms might be found that corresponded to such a description but that would probably not be specifically identical (see Rodella). Bienstock himself refers to his organism as *B. putrificus* and as *Putrificus* (1899).

A SUGGESTED CLASSIFICATION OF THE ANAEROBIC BACTERIA

PHYLUM 1. *Bacteria* (Nov. phyl.): Simple one-celled plants that multiply typically by binary fission and occasionally by budding. They show no form of sexual multiplication. They rarely contain cellulose and do not contain chlorophyll or phycocyanin.

CLASS 1. *Eubacterieae.*

CLASS 2. (*Myxobacterieae, Bacteria* which join to form a complex fruiting body (see Vahle, p. 196)).

CLASS 1.	*Eubacterieae*: *Bacteria* which do not form a complex fruiting body.
ORDER 1.	*Eubacteriales*.
ORDER 2.	(*Thiobacteriales*, sulphur bacteria.)
ORDER 3.	(*Chlamydobacteriales*, iron and manganese bacteria.)
ORDER 1.	*Eubacteriales*: *Eubacterieae* whose cells are never in sheathed filaments. Conidia not observed. Free iron, sulphur, or bacteriopurpurin never present. Multiplication always occurs by transverse fission. (Committee)
FAMILY 6 (?)	*Clostridiaceae* (nov. fam.): *Eubacteriales* that are rod-like, not spiral, that will not grow within 7 mm. of the surface of a shaft of clear tissue-free agar medium contained in a tube 12 millimeters or more in diameter, incubated in air, in which they are able to grow in the depths. They may or may not possess peritrichial flagella, they may or may not form endospores. Most members of the group are characterized by their energetic catalytic action on proteins or on carbohydrates or on both of these types of substances.
SUBFAMILY 1.	*Clostridioideae* (nov. subfam.): *Clostridiaceae* which on meat medium produce after twenty days' incubation under vaseline at 37° a reaction of pH 7.0 or a more acid reaction, the reaction being read after the culture has been boiled. Type genus *Rivoltillus* (nov. gen.), the vibrion septique type as described by Heller.
SUBFAMILY 2.	*Putrificoideae* (nov. subfam.): *Clostridiaceae* which on meat medium produce after twenty days' incubation at 37° under vaseline a reaction of pH 7.1 or a more alkaline reaction, the reaction being read after the culture has been boiled. Type genus *Metchnikovillus* (nov. gen.), the sporogenes types as defined in the description of *Bacillus sporogenes*, described by the Medical Research Committee as Metchnikoff's type A.

SUMMARY

1. Morphological criteria cannot be used in classifying the higher groups of anaerobes.

2. The anaerobic rods may logically be placed in a common family on the basis of the physiological character of sensitiveness to free oxygen.

3. This family may be subdivided into two sub-families on the basis of chemical action on carbohydrates and proteins.

4. The divisions "tribe" and "sub-tribe" may well be left open for future organization.

5. The old conception of species in the anaerobic group corresponds to the general systematists' conception of genera. Generic characters may be based chiefly on qualitative behavior on ordinary media. Pathogenesis and general morphology may be used as auxilliary generic characters.

6. Specific characters may be based on sugar fermentations, on quantitative chemical action, on the morphology of colonies and to some extent on the morphology of individuals.

7. We are not as yet ready for extensive biometric determinations in studying many of the anaerobic groups and must temporarily adopt a more easily performed technique for the distinction of these organisms.

8. A classification of the anaerobic group is proposed whose details are to be elaborated in a following paper.

I wish to extend my hearty thanks to Dr. K. F. Meyer and to Dr. Harvey M. Hall for reading the manuscript of this paper and for making many wise and helpful suggestions which have been followed in its compilation.

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PHYLOGENETIC POSITION OF THE BACTERIA

XVIII

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PHYLOGENETIC POSITION OF THE BACTERIA[1]

HILDA HEMPL HELLER

The subject of the phylogenetic position of the bacteria has been approached by many students. Early workers came to no more diverse conclusions than do modern ones. Some investigators, for example NÄGELI (24) and GÖTSCHLICH (14), have placed the bacteria with the fungi, while COHN (9), MIGULA (22), and SACHS (26) placed them with the algae. The early workers who assigned the bacteria to the fungi did so because both fungi and bacteria lack chlorophyll, and may thus be regarded as similarly degenerate algae, and because there are genera such as *Corynebacterium*, *Actinomyces*, *Streptothrix*, and *Oidium*, that may well be regarded as transitional forms. Classifiers of the fungi have not sufficiently emphasized the fact that in a group where chlorophyll is absent there is no compelling reason for presuming that the simpler forms, the bacteria, were descended from the higher ones, as the workers thought who considered them as directly descended from the algae. Even DE BARY (1), although he uses NÄGELI's name "Schizomycetes" (fission fungi) for the bacteria, insists that they are not fungi, nor closely related to or descended from fungi.

The reason for classing the bacteria as a subordinate group of the algae has usually been the exceedingly close morphological resemblance of the higher bacteria to the blue-green algae (Cyanophyceae or Myxophyceae). COHN was the first to emphasize the relationship between these groups. The Cyanophyceae were long thought to be the most simple autotrophic forms. More modern systematists have separated the blue-green algae from those with sexual reproduction, and have united them with the bacteria. Thus ENGLER (12), in his second phylum Schizophyta, included only the two classes Schizomycetes and Schizophyceae; WARMING (28) made a similar division of his class Schizophyta; while BESSEY (3) in his phylum Myxophyceae, class Archiplastideae

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[1] From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco.

(blue-green algae without nuclear membrane), placed the Bacteriales as an order coordinate with two orders of the blue-green algae. BÜTSCHLI (6) and KLEBS (19) emphasized the common characters of the bacteria and the protozoa.

Today the question is apparently no nearer a solution than it was forty years ago. The various views are based on the consideration of different life manifestations. Close relationship between bacteria and protozoa, however, is no longer emphasized. The principal views held today are three: (1) that the bacteria are members of the group of fungi, (2) that they are derived from or closely related to the Cyanophyceae, and (3) that they are the primitive forms from which fungi and algae are derived. The first two opinions are held by morphologists. Those who have regarded the manner of division and sporulation, the external characters of the organism so to speak, and those who hold that bacteria possess a small oval or round nucleus, have allied the bacteria with the fungi. Others who have studied their nuclear structure have allied them with the Cyanophyceae. Chemists and general students of evolution have recently considered them as ancestors of the other groups.

The morphological field has been reviewed carefully by MEYER (21), who holds that the closest affinities of the bacteria are with the fungi. The bacterial nucleus, according to his conception, is similar to the nucleus of the fungi. SWELLENGREBEL (27), GARDNER (13), and DOBELL (11) have observed structures, which they believe to be nuclei, that have marked resemblance to the chromophyll portion of the central bodies of the Cyanophyceae. GUILLIERMOND (15) and a number of other workers hold the bacterial nucleus to be "chromidial" or finely distributed in the cytoplasm. DOBELL finds structures resembling all these types, which he holds to be nuclei, and he believes the bacteria to be related most closely to the Cyanophyceae. WEST (29) described a blue-green alga, *Myxobactron*, which shows no differentiation of its protoplasm. PARAVICINI (25) has recently described minute compact structures that he believes to be nuclei.

JENSEN (18) rearranged the bacteria on a chemical basis, and defined their relation to the algae, fungi, and protozoa, presuming that the earth was dark when life began, and that chlorophyll-free bacteria, probably those capable of oxidizing methane, were the earliest forms of life with which we are familiar today. JENSEN derived the blue-green algae from the sulphur bacteria, the fungi from the oxidizing bacteria by way of the Actinomycetes, and the higher bacteria from the earliest nitrogen-reducing organisms. KLIGLER (20) was also of the opinion that bacteria may well have been the earliest forms of life, and he placed the methane-oxidizing type at the base of his tree. BREED, CONN, and BAKER (4) pointed out that there is no proof that the world was dark when life began; that in case it was light the ancestors of the blue-green algae or of the phototrophic pigment bacteria, which use sunlight to metabolize organic substances, may have been the most primitive forms; or that the most primitive form may be entirely unknown to us. Thus we see that because of the discovery of the existence of autotrophic bacteria the old question of the origin of the bacterial group is again open.

When the synthesis of inorganic substances into organic material was thought to be possible only by the aid of chlorophyll, the natural trend of evolutionary reasoning led to the derivation of other forms of life from simple chlorophyll-containing ones. Bacteria apparently are simpler than the most simple chlorophyll-bearing algae. They were therefore thought to be degenerate. Workers who saw in them affinities with the chlorophyll-free fungi were not careful to state what their relationship with the fungi really might have been. The reader is usually left with the impression that they are in an intermediate position or related to the higher fungi. MEYER, who excluded the *Thiobacteria*, *Chlamydobacteria*, and *Myxobacteria* from his Eubacteria or bacteria proper, placed his group as the second class of the Eumycetes next to the Phycomycetes or algal fungi. CLAYPOLE (7) considered both bacteria and fungi to be derived from the leptothrix-tuberculosis group.

A rather suprising paper has recently appeared by BERGSTRAND (2), who has observed the budding and branching of *Corynebacterium* and other forms, and believes that the bacteria are closely related to the fungi. Budding and binary fission are not so different in their nature that they should be considered very important characters. One genus of yeasts, the *Schizosaccharomyces*, divide as do the bacteria. Apparently typhoid bacilli may either bud or divide by fission HORT (16). Upon this one character of budding, BERGSTRAND lays so much emphasis that he refuses to consider other characters, also morphological, which show similarity between the bacteria and other forms: "To discuss further the eventual relationship of Cyanophyceae to bacteria does not seem necessary, because any such theory would appear false at the moment that it became clear that bacteria are more closely related to fungi, as I shall show." It must be noted that, like MEYER, BERGSTRAND excludes from his bacterial group the higher bacteria which do not resemble the fungi as much as they do the algae. One would be equally justified in naming as bacteria all the chlorophyll-free rods except the branching and budding ones. BERGSTRAND defines the bacteria as Fungi Imperfecti. The Fungi Imperfecti are an entirely artificial group comprising fungi that have not developed sexual characters, those that have lost such characters, and those that have not been studied sufficiently to determine their true relationships. BERGSTRAND concludes that bacteria are to be regarded as Fungi Imperfecti that have developed through the reduction of higher forms, and not as lowly primordial organisms to be placed at the very beginning of the organic world. An example of his logic is as follows: "Of course if one regards bacteria as Fungi Imperfecti one cannot accept the theory that the chromatin is spread diffusely in the cell body, because this assumes it would seem a much lower developmental stage."

It is not the intention of this paper to criticize workers for connecting bacteria with fungi because of morphologic relationships between the two groups. BERGSTRAND'S observations serve to strengthen the tie between the fungi and the bacteria, but the lightness with which he proposes the degeneracy of the latter forms from the former is a novel process to comparative biological reasoning. The trend of evolution is rarely in the direction of degeneracy. Degeneracy occurs as a consequence of a parasitic habit or because of abundant food supply. It is usually accompanied by vestigial traces of a former complexity. The characters which the bacteria and fungi have in common are not manifestly vestigial in the bacteria. The supposed loss of sexual characters among the fungi has been attributed to their change from water forms to air forms, but bacteria are not air forms. The theory of the degeneration of the bacteria from the algae was a very peculiar one, imposed by ignorance of certain primitive bacteria. It is now known that bacteria exist which are autotrophic and can secure growth energy from inorganic carbon, so that their lack of chlorophyll is no longer a reason for considering them degenerated from the chlorophyll-containing forms. The existence of autotrophic fungi, to my knowledge, has never been demonstrated.

There is a simple group, therefore, the members of which are autotrophic; and two diverse complex groups, one of which (the fungi) is not autotrophic and may not be homogeneous. Both of these complex groups show marked resemblances to the simple one. JENSEN'S scheme, which derives both of them from the simple one, is not to be lightly thrown aside. It coincides too well with the general scheme of evolution. We may, if we wish, consider the question entirely open, but nomenclature and classification should be so formulated that they do not deliberately mislead the amateur on the subject of these relationships. Formerly the tendency in botanical classification was to make a treelike structure, throwing groups together that had but superficial resemblances, but classifiers today are more prone to refuse to indicate relationships where descent is not fairly certain, and to group the plants in phyla like the zoological phyla, whose connections may or may not be understood.

The bacteria, fungi, and blue-green algae, therefore, may be all in one phylum, or may be placed in three separate phyla, but to place the bacteria with either fungi or Cyanophyceae is inconsistent, because it leaves out of consideration the third group which may be equally related to the bacteria. Probably the trend of classification would favor the separation of these groups into three separate phyla, for to place the fungi and Cyanophyceae together is rather stretching the limits of the botanist's conception of a phylum. Moreover, in view of the existing divergent opinions, a classification that does not commit one on the subject of these relationships is preferable. A name for the phylum that is to contain the bacteria only should not indicate for them a subordinate position in another group as does the name "Schizomycetes," proposed by Nägeli (23) in 1857 for a mongrel group which contained bacteria, sporozoa, and oscillaria, a group whose affinities he hesitated to suggest. The connotation of this term has always been "fission fungi," and its German form "Spaltpilze" has been widely used. And yet Buchanan (5) finds it entirely appropriate and valid and proceeds to place his Schizomycetes with the Cyanophyceae. Article 51, division 4, of the Vienna rules (17) considers the name Schizomycetes as invalid. "Everyone should refuse to admit a name . . . . when the group which it designates embraces elements altogether incoherent, or when it becomes a permanent source of confusion and error."

We should choose for the bacterial phylum a name that will immediately be understood by the non-professional worker. Names like *Phytozoidia* Perty of course are objectionable. *Vibrio* Ehrenberg probably included certain infusoria as well as bacteria. *Vibrionia* Cohn did not include forms later studied by that author. *Bacteria* Cohn (8) probably included all the forms that we today call bacteria except *Beggiatoa*, and it did not include members of other groups. As the Committee of the Society of American Bacteriologists (10) places 1880 as the date at which considerations of priority are to commence, we are free to choose from among these names. Bacteria implies no relationship to other groups. It is otherwise highly suitable because it is understood by laymen and is short and euphonious. The following was Cohn's conception of the group: "Die Bacterien sind chlorophyllose Zellen von kugeliger, oblonger oder cylindrischer, mitunter gedrehter oder gekrümmter Gestalt, welche ausschliesslich durch Querteilung sich vermehren, und entweder isoliert oder in Zellfamilien vegetieren."

In consideration of the fact that no relationship of the bacteria to other groups has been generally accepted the following phylum is proposed:

Bacteria (nov. phyl.).—Simple one-celled plants that multiply typically by binary fission and occasionally by budding. They show no form of sexual multiplication. They rarely contain cellulose and do not contain chlorophyll or phycocyanin.

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XI4

SOME SUGGESTIONS CONCERNING THE BACTERIOLOGICAL DIAGNOSIS OF HUMAN BOTULISM

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SOME SUGGESTIONS CONCERNING THE BACTERIOLOGICAL DIAGNOSIS OF HUMAN BOTULISM.[1]

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Considerations Prompting Bacteriological Study.

In the course of an analysis of a fairly large number of outbreaks of botulism in man reported during the last 10 years, it was noted that the diagnosis was frequently based on clinical symptoms only. Sometimes it was possible to demonstrate the botulism toxin or the organisms in the causative food. In numerous instances, however, the remnants of the suspected food were evidently not available or were examined by a chemist instead of a bacteriologist. From a medico-legal, and also from an epidemiological, standpoint, the diagnosis could therefore be questioned, and the statistician attempting to unravel the all-embracing diagnosis "ptomaine or food poisoning" finds little definite information in the published reports. If one recalls, moreover, that botulism can be mistaken by the inexperienced person for methyl-alcohol poisoning, encephalitis lethargica, or cerebrospinal syphilis, it is obviously important to conduct careful necropsies on such cases and to determine either by cultural or histological studies the true nature of the disease. In connection with the latter procedure it must be said that the characteristic thrombi or prethrombotic stages in the arteries and veins of the meninges and brain originally described by Ophüls (1) may not be present, and a microscopic study alone may therefore fail to render a diagnosis. Bacteriologic studies of the tissues of fatal cases have been made in a few instances. V. Ermengem (2), Ornstein (3), and Graham (4) report the demonstration of *B. botulinus* in the spleen of fatal botulism cases. Some writers also recommend a cultural study of the intestinal contents at autopsy, but nothing is said regarding the possibility of finding *B. botulinus* in the stools of clinical cases.

Theoretically, at least, stool examinations appear to be valuable, inasmuch as numerous observers have found this organism in the excreta of animals which ingested food spontaneously or experimentally contaminated with the poison and spores of the organism Constipation is almost a constant manifestation of botulism and is naturally conducive to the persistence of the organism for a considerable period in the alimentary canal of man and animals. This must be particularly the case in those instances in which a bowel movement can not be procured, in spite of all medication, until the 10th or even the 16th day, as reported by Schumacher (5).[1]

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These and other considerations to be discussed elsewhere prompted us to study bacteriologically some cases of botulism which came to our notice during the last 12 months. The findings thus far made are suggestive and are reported in order that other workers may amplify our observations when the occasion arises.

Methods Employed in Culturing Tissues or Stool Specimens for B. botulinus.

Portions of the organs are ground in a mortar with sea sand and emulsified with saline. Stool specimens are diluted with saline until the formed portions are finely divided. The emulsions are placed in 250–c. c. culture flasks and heated for one hour at 60° C. They are then mixed with 100 c. c. sterile beef heart medium, which consists of one part of ground beef heart and two parts of peptic digest broth of a reaction P_H 7.4. The mixture is stratified with oil, or, better, with vaseline. The flasks, which are closed with rubber stoppers and sealed with Imperial or Major's glue, are exhausted of air as completely as possible. After incubation for from 10 to 30 days at 37° C., the centrifugalized supernatant fluid is administered, in 1– to 2–c. c. doses, to guinea pigs. The presence of *B. botulinus* toxin is definitely ascertained by a neutralization test with known type A, and B, *B. botulinus* antitoxic sera. Isolation of *B. botulinus* is accomplished by fractional heating, enrichment, and deep cultivation in liver-peptone agar. Heating of the emulsions at 60° C. for one to two hours alone insures the possibility of obtaining proper cultures, as is shown in the case reports.

Report of Cases.

Richmond, Calif., outbreak (Feb. 25, 1920).—*B. botulinus* type A, and *B. botulinus* type A toxin were demonstrated in a can of olive relish, responsible for one fatal case. Anaerobic cultures from the spleen of the patient (25 grams), liver (20 grams), lung (10 grams), kidneys (9 grams), mucus from ileum (5 grams), and jejunum were negative for *B. botulinus.* The intestinal wall was not cultured.

Florence, Ariz., outbreak (May, 1920).—Canned beets were suspected as the causative food. For a bacteriological examination, the spleen (weight 242 grams), a portion of the jejunum, and the brain of Ch. R., who died on May 19, 1920, were available.

Cultures of the spleen (30 grams) and chyme (5 c. c.) of the intestinal loop demonstrated *B. welchii*, *B. sporogenes*, and *B. bifermentans.*

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[1] References:
(1) Arch. Int. Med. 1914, 14, p. 589.
(2) Ztschr. f. Hyg. u. Infektionskr. 1897, *26*, p. 4.
(3) Ztschr. f. Chemotherap. Orig., 1913, *1*, p. 458.
(4) McCaskey: Am. Jour. Med. Sc. 1919, *158*, p. 57.
(5) Munch. Med. Wchnschr. 1913, *60*, p. 124.

A specimen consisting of 4 grams of macerated jejunal wall gave a culture of *B. botulinus*, type B, associated with *B. tertius*, *B. welchii*, *B. sporogenes*, *B. tetano morphus*, and two other unidentified anaerobes. The strain of *B. botulinus* was isolated in pure culture. The remaining portion of the intestinal wall, which had been kept in the ice chest for four weeks, had undergone autolysis and decomposition, but when cultured, *B. botulinus*, type B, was isolated. Sections of the brain revealed definite prethrombotic stages in the blood vessels of the brain. Cultures of the brain revealed cocci and gram negative aerobic rods.

Oakland, Calif., outbreak (October, 1920).—Canned spinach was suspected as the causative food. Available for a bacteriological study were some stool specimens collected from Miss A. R., who recovered from the disease. The responsible meal was consumed on October 14, 1920; the first symptoms were noted on the 16th; intestinal washings were obtained on October 20, and a formed stool was obtained on October 21, 1920. Six specimens of 75 c. c. each of the intestinal washings were heated for one hour at 60° C. and cultured; five contained *B. botulinus*, type A. Five specimens of 75 c. c. each were cultured in an unheated state. These cultures, on repeated tests, were negative for *B. botulinus*. Three samples of 50 grams of solid stool were emulsified in saline, heated at 60° C. for one hour and cultured. Only one sample gave a culture of *B. botulinus*. Three unheated specimens of the same sample were negative. *B. botulinus* was, therefore, present in six stool specimens collected on the sixth and seventh days, respectively, after the consumption of the causative meal.

Grand Rapids, Mich., outbreak (January, 1921).—Canned spinach was suspected as the causative food. Through the courtesy of Dr. Merrill Wells the intestinal washing (enema) of Miss H. was collected on the eleventh day and made available on the seventeenth day after the consumption of the infected meal. One flask out of five stool specimens of 50 c. c. each, which had been heated to 60° C. and cultured, contained, on the tenth day of incubation, *B. botulinus* toxin, type A. The isolation of the organism in pure culture is in progress. Two unheated specimens were negative. *B. botulinus* was, therefore, present in a stool specimen on the eleventh day after the causative meal had been consumed.

Comment.

The foregoing observations indicate that the spores of *B. botulinus*, when presumably ingested in the poisonous food, may remain in the intestinal canal and may be eliminated in the stools of typical botulism cases. Several important problems suggest themselves for immediate experimental study or investigations on human cases of this disease; the following points deserve particular consideration: (1) Determination of the average period of fecal discharge of *B. botulinus* spores in severe and mild cases of botulism; (2) quantitative estimation of the eliminated spores per 1 or 10 grams of enema or formed stool; (3) quantitative comparison of the spore content of the causative food and that of the stools; (4) testing of filtered stool suspensions, on guinea pigs, for the presence of toxin; and (5) testing for *B. botulinus* spores the stools of normal human beings who eat raw fruit or vegetables and live in districts in which the organism is quite common in the soil.

These investigations would undoubtedly contribute information as to the possible pathogenicity of *B. botulinus* spores as suggested by Orr (6), Edmondson, Giltner, and Thom (7), and others. *B. botulinus* possesses a noteworthy degree of growth adaptability, and it is possible that the spores can germinate in the paretic intestinal tube and form toxin. Some personal observations on spontaneously diseased domesticated animals justify this suggestion. It appears also of importance to know if botulism convalescents can remain "spore carriers" and, as such, assist in the progressive pollution of the earth with dangerous bacteria. The diagnostic value of the demonstration of *B. botulinus* spores can only be accepted when repeated tests on normal stool samples have demonstrated an absence of this organism. An experimental study of the problem mentioned under (5) in the preceding paragraph is in progress. The examination of numerous sewage samples of urban and rural origin has thus far failed to give positive *B. botulinus* cultures, and we therefore feel confident that the stool test will be of practical value. However, it remains to be demonstrated as to the number of days a botulism patient is capable of discharging *B. botulinus* spores. The observations made by Thom, Edmondson, and Giltner (8), and others on guinea pigs strengthen our belief that the spores may be demonstrated only in the fecal remnants of the causative meal. Inasmuch as the discharge of this material is quite often delayed for many days, on account of the intestinal paresis, positive findings may be recorded for two, perhaps even three, weeks.

A quantitative estimation of the spores in the stool samples or in the causative food offers no technical difficulties. For example, in one of the recent outbreaks the spinach responsible contained *B. botulinus* spores in practically a pure state. Shake cultures and particularly those in dried liver agar gave colonies which could be readily counted.

The finding of *B. botulinus* spores in the jejunal wall, but not in the chyme, of the particular intestinal loop mentioned may be merely accidental or may vaguely support the recently advanced, but rather fanciful, conception (9) that "*B. botulinus* when taken into the human system lodges in the digestive tract, and the toxins produced there spread over the body." It is our intention to discuss this phase of botulism elsewhere in detail; nevertheless, the diagnostic significance should be emphasized. We had recently occasion to study, in cooperation with Dr. L. R. Vawter, of Reno, Nev., a cattle disease in which *B. botulinus* apparently exhibited invasive properties. Invariably the organism was isolated from the inflamed duodenum and jejunum, the liver, mesenteric lymph-nodes, etc.

It is noteworthy that our two attempts to isolate *B. botulinus* from the spleen were not successful. These results may, in part, be due to the fact that post-mortem invasion was made impossible by the early removal and careful preservation of the tissues after death.[1]

Summary.

B. botulinus, type B, has been isolated from the jejunal wall of a case of botulism fatal on the fifth day of the disease. Spleen cultures in two instances were negative for *B. botulinus*. Stool specimens of two clinical cases of botulism, obtained from two different outbreaks, contained *B. botulinus*, type A, on the sixth, seventh, and eleventh day, repectively, after the consumption of the causative meal. The methods of tissue and stool cultures are described. The importance of culturing the stools and tissues of all clinical cases of botulism is evident

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[1] References:

(6) Proc. Soc. Exp. Biol. and Medicine, 1919, 17, p. 47.
(7) Arch. Int. Med., 1920, 26, p. 357.
(8) Jour. Am. Med. Assn., 1919, 73, p. 911.
(9) Boston Med. & Surg. Jour., 1920, July 29, 183, p. 139.

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EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS

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The part played by the human carrier in the spread of typhoid fever is well known. The experience of various health departments shows that today flies, food (exclusive of milk), privies and sewage are relatively small factors in the dissemination of this disease. In the state of Massachusetts, according to O'Donnell,[1] few cases are due to water, especially in recent years, and in general no municipal supply is considered as a dangerous source of typhoid infection. Contact with true typhoid has been responsible for a few cases in homes and neighborhoods, while carriers have been proved to be responsible for 41.6% of cases of milkborne typhoid from 1915 to 1918, inclusive. It is therefore evident that carriers are a larger factor in the spread of typhoid than at present realized and are probably also the cause of an appreciable number of typhoid cases of unknown origin. This statement is supported by the fact that from 1915-1919, 14 carriers caused 249 cases of typhoid fever. Similar observations have been made by Chesley [2] and his associates, who traced 213 cases of typhoid fever in Minnesota to 30 carriers, or an average of 7 cases each. In California also the persistence of typhoid fever is definitely linked with carriers. Certain data collected in the army camps (Camp Dix) and in the Expeditionary forces clearly suggest that, in some instances, this mode of infection was responsible for small epidemics. Moreover, Garbat [3] has recently estimated that 55% of all typhoid may be traced directly or indirectly to carriers. The general reduction of typhoid in the United States has enabled the health authorities to devote more attention to the prevention of contact and carrier cases. A statement by Meader [4] summarizes the present situation in an excellent manner. He says: "By far the most difficult source of infection to trace and the most baffling to control is the typhoid carrier, and methods for the cure of typhoid carriers are among the most needy research problems."

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[1] Am. Jour. Public Health, 1920, 10, p. 517.
[2] Jour. Am. Med. Assn., 1917, 68, p. 1882
[3] Ibid., 1916, 67, p. 1493.
[4] Mod. Med., 1920, 2, p. 244.

An elimination of typhoid fever can obviously only be expected when the problem dealing with the prevention and cure of carriers has been solved. Förster [5] has stated that if we can cure carriers we can get rid of typhoid fever. Unfortunately, it must be admitted that, hitherto, no certain method has been devised for rendering carriers typhoid-free. Biologic, as well as chemical, preparations have been applied to a limited number of intestinal carriers without any constant result. Surgical treatment, while not perfect, is at present considered the best available.

Since it is possible to produce in rabbits a condition similar to the carrier state in man, the new science of chemotherapy of bacterial infections has been tried extensively. The success so far achieved in rendering the rabbit carriers typhoid-free can be considered encouraging, but in no way striking. In all the experiments pertaining to the typhoid problem, it appears that therapeutic attempts have been made on a process, the pathogenesis of which is unknown, or in its best forn very superficially investigated. If the numerous tests had been crowned with success, which, as already stated has not been the case, one could attribute the result more to chance than to a well planned systematic investigation. Therapeutic studies must be preceded by an experimental analysis of the factors leading to a persistence of the typhoid bacilli in certain tissues, and with it to the carrier state. It is unquestionably true that the prevention of typhoid carriers can only be accomplished when the pathogenesis of this condition is known in detail.

These and similar considerations suggested in 1915 a systematic study of the typhoid infection in various laboratory animals. No attempt was made in using the words of Metchnikoff and Besredka,[6] "de resoudre le probléme de la fiévre typhoide si difficult et si compliqué." It was intended to place our knowledge concerning typhoid infection in rabbits on a broader basis. The subject was approached by making use of the well-known gall-bladder infections readily produced in these animals. It was soon realized that other laboratory animals must be included in the scope of the experimental observations. In dealing with so intricate a problem, on which a great deal had been written and which had been considered experimentally from so many different angles, the best working plan consisted in treating the typhoid bacillus and its allied organisms as living entities with an adaptive organization and to follow them step by step in their passage through the animal body. The various phases of this investigation thus far completed with the aid of my collaborators will be summarized in a series of papers which will be submitted for publication as rapidly as other duties permit the undertaking.

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[5] München. med. Wchnschr., 1908, 55, p. 1.

[6] Ann. de l'Inst. Pasteur., 1911, 25, p. 193.

The papers in preparation cover the following subjects: The Optimum H-ion Concentration for Growth of B. typhosus and B. paratyphosus A and B (paper 2); Do Carrier Strains differ from Strains Recently Isolated from Ordinary Typhoid Cases? (paper 3); A Comparative Study of the Infections Produced by Intravenous Injections of Typhoid, Paratyphoid A and B bacilli in Normal and Immunized Rabbits (paper 4); The Mechanism of Gallbladder Infection in Laboratory Animals (5); The Reaction and the Physiology of the Gallbladder and Hepatic Duct Bile of Laboratory Animals (6); The Bacteriostatic and Germicidal Properties of Bile (7); The Influence of the H-ion Concentration on the Growth of B. typhosus in Mediums Containing Bile or Bile Salts (8). Subsequent papers are planned to deal with: Feeding Experiments on Rabbits with B. typhosus and B. paratyphosus; Diet, Starvation and Immunization as Factors in the Production of the Intestinal Carrier State in the Rabbit; The Physical Properties of the Inoculum in the Production of Carriers; The Examination of Animal Stools as an Aid in the Diagnosis of the Carrier State; Renal Carriers in Rabbits; The Fate of B. typhosus and B. paratyphosus B Intravenously Inoculated into Guinea-Pigs, Cats, Rats and Monkeys; Typhoid and Paratyphoid A and B Infections Produced in Guinea-Pigs by Gallbladder Injections; Feedings of B. typhosus in Normal Guinea-Pigs and Those Suffering from "Deficiency" Disease; A Study of the Tissues Obtained from Experimental Carriers; and finally, A Correlation of the Experimental Results with the Facts Available from the Study of Human Carriers.

THE OPTIMUM HYDROGEN-ION CONCENTRATION FOR THE GROWTH OF B. TYPHOSUS, AND B. PARATYPHOSUS A AND B

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. II

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It is known that B. typhosus and the allied organisms of this group grow best in a neutral or slightly acid medium. The present study was undertaken to determine the probable range of growth and the optimum H-ion concentration.

There are two methods commonly employed to measure the H-ion concentration of a solution. The first is by means of the H-electrode, the second is the colorimetric method. For very accurate work, in which slight changes in H-ion must be determined, or in which the greatest possible accuracy is to be attained, the first method of measuring H-ion concentration is generally used. However, when the changes in H-ion concentration extend over a broad range, and when the method of experiment calls for a large number of determinations of only relative accuracy, the second method is to be preferred. This is particularly true when the solutions to be tested are more or less complex and the salt and protein content interferes with the electrometric determinations.

The ability of certain solutions to resist changes in H-ion concentration is called its buffer action. The body fluids, ordinary Witte's peptone solution, solutions of weak acids, such as phosphates, borates and acetates, and many others, exhibit this property. This behavior of certain substances in solution has been used by several investigators in the preparation of solutions of known H-ion concentration. Sörensen[1] carefully studied such a set, as did Walpole[2] and Palitsch[3]; Clark and Lubs[4] have recently modified these sets slightly. In our work, we have found it necessary to prepare these buffer solutions in a range extend-

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[1] Biochem. Ztschr., 1907, 7, p. 131.

[2] Biochem. Jour., 1910, 5, p. 207.

[3] Biochem. Ztschr., 1912, 47, p. 1.

[4] Jour. Bacteriol., 1917, 2, p. 1.

ing from $P_H + 4.0$ to $P_H + 9.0$. These solutions were carefully prepared[5] and checked (through the kindness of Dr. Schmidt) with a H-electrode.

Up to recent years one of the main objections to the use of the colorimetric method for the determination of H-ion concentration in bacteriologic work was the scarcity of brilliant indicators covering the entire range of H-ion concentration. Phenolphthalein, an indicator most commonly used, has a range on the alkaline side from $P_H + 8.0$ to $P_H + 10.0$. Litmus it not sensitive or brilliant enough for colorimetric work. Methyl red has been used and has proved very satisfactory. It passes from its full acid color into its alkaline color as the H-ion concentration falls from $P_H + 4.8$ to $P_H + 6.4$. Through the work of Clark and Lubs,[4] however, several new and brilliant indicators have become available for this work. Methyl red, bromthymol blue, cresol red and thymol blue were the indicators most generally used in this work.

There has been a great deal of discussion relative to the death point and its relation to the H + ion concentration. Bruenn,[6] using mixtures of lactates as well as acetates of a definite H + ion concentration, came to the conclusion that the disinfection which takes place at high H + ion concentrations depends on the $P_H +$. He found that the death point of B. coli was between P_H 4.7 and 4.4; for B. typhosus between $P_H + 5.6$ 5.0-4.7. We have not noted quite as large a range in our experiments, as will be indicated later. Winslow and Lockridge[7] have shown that typhoid bacilli are a little less than half as resistant as B. coli to dilute acids. Using HCl and H_2SO_4, the toxicity depends on the H + ion concentration; the undissociated part of the molecule exerts no toxic effect. Organic acids, such as benzoic or acetic acids, are toxic for these organisms in strengths in which they are only slightly dissociated. The toxicity is the result of the undissociated part of the molecule. The accumulation of similar organic acids might account for the phenomena observed by Clark, that the final H + ion concentration is lower in more highly buffered mediums.

In order to determine the degree of acidity or alkalinity necessary to inhibit B. coli in the animal body, Shohl and Janney[8] studied the growth of these organisms in urine at varying H + ion concentrations. They showed that these organisms were inhibited in their growth at a P_H + of 4.6-5.0 on the acid side, and from P_H + 9.2-9.6 on the alkaline side. The optimum growth for B. coli was between 6.0-7.0. They also tested the Rawlings strain of B. typhosus (our strain 6) and state that the typhoid bacillus has narrower limits, showing no growth at P_H + 5.0 on the acid side, or P_H + 8.8 on the alkaline side. Quite recently Winslow, Kligler and Rothberg[9] found that B. typhosus (Rawlings' strain) produces, inside of 24-96 hours, in hexoses an acid reaction of P_H + 5.5-5.0, which remains practically constant.

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[5] We are indebted to Mr. P. Edson for the preparation and standardization of these solutions.

[6] Jour. Urology, 1917, 1, p. 211.

[7] Jour. Infect. Dis., 1906, 3, p. 547.

[8] Jour. Urology, 1917, 1, p. 211.

TABLE 1

HYDROGEN-ION CONCENTRATION OF MIXTURES OF SALT-FREE BROTH, HYDROCHLORIC ACID OR SODIUM HYDROXIDE AND PHOSPHATES

10 c c broth + 2 c c 5M phosphates + HCl or NAOH + H_2O = 13 c c

Tube No.	0.1 n NaOH, C c	0.1 n HCl, C c	0.5 n KH_2PO_4, C c	0.5 n Na_2HPO_4, C c	Hydrogen-ion Concentration of Mixture, P_H
		P_H of Medium = 7.4			
	3.0	...	...	...	9.0
1	2.4	...	...	2	8.8
2	2.4	...	...	2	8.6
3	2.0	...	0.04	1.96	8.4
4	1.3	...	0.1	1.9	8.2
5	1.0	...	0.12	1.88	8.0
6	0.6	...	0.14	1.86	7.8
7	...	...	0.2	1.8	7.6
8	...	...	0.4	1.6	7.4
9	...	...	0.7	1.3	7.2
10	...	0.5	0.8	1.2	7.0
11	...	0.6	1.0	1.0	6.8
12	...	0.8	1.2	0.8	6.6
13	...	1.1	1.45	0.55	6.4
14	...	1.6	1.7	0.3	6.0
		P_H of Medium = 6.4			
15	...	0.6	1.7	0.3	6.0
16	...	0.8	1.8	0.2	5.8
17	...	1.0	1.9	0.1	5.4
18	...	1.7	2	...	5.0
19	...	2.1	2	...	4.8
20	...	2.4	...	...	4.3

The exact effect of variations in the H + ion concentration of culture mediums on B. typhosus has not been investigated extensively. It was thought advisable to investigate this problem before attempting other work in which variations in the H + ion concentration are concerned. Moreover, it will be evident from the observations reported in subsequent papers (6, 7 and 8) of this series, that a detailed knowledge of the H-ion requirements of an organism is absolutely essential before an explanation can be attempted relative to the behavior of a bacterium in the body fluids or the secretions of an animal.

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[9] Jour. Bacteriol., 1919, 4, p. 471.

Technic.—Preparation of Mediums: The mediums were prepared by allowing 500 gm. of lean veal to infuse in 1000 c c of tap water on ice over night. This infusion was boiled for 20 minutes, strained through cheese cloth and allowed to cool. The fat was removed by filtration, 1% "Difco" peptone was added, and the medium adjusted to a reaction of $P_H + 7.4$. After stabilization in the Arnold for 30 minutes, the broth was filtered and sterilized by fractional sterilization in live steam for 3 consecutive days. This "salt free medium" was made in large quantities. The same lot of medium was used for the entire series of experiments.

TABLE 2

INFLUENCE OF SALT CONCENTRATION ON THE GROWTH OF B. TYPHOSUS
INCUBATION 18 HOURS AT 37 C.

P_H 7.0 Molal Concentration	Growth			
	NaCl		KCl	
	Kearney	Blair	Kearney	Blair
1.0	∓	∓	—	—
0.5	++	++	++	++
0.3	+++	+++	+++	+++
0.1	+++	+++	+++	+++
0.06	+++	+++	+++	+++
0.04	+++	+++	+++	+++
0.02	+++	+++	+++	+++
Salt free broth	+++	+++	+++	+++

TABLE 3

INFLUENCE OF PHOSPHATES ON B. TYPHOSUS BROTH AND PHOSPHATE MIXTURE
(7 PARTS Na_2HPO_4 + 3 PARTS KH_2PO_4)

+ 0.1 c c of 1: 10,000 20-hour broth culture + distilled H_2O = 5 c c

Strain								
Kearney			Jacobs			Blair		
Tube Number	Molal Concentration of Phosphate	Growth	Tube Number	Molal Concentration	Growth	Tube Number	Molal Concentration	Growth
20	0.4	∓	25	0.4	∓	30	0.4	∓
21	0.3	++	26	0.3	++	31	0.3	++
22	0.2	+++	27	0.2	+++	32	0.2	+++
23	0.1	+++	28	0.1	+++	33	0.1	+++
24 Salt-free broth	...	+++	29 Salt-free broth	...	+++	34 Salt-free broth	...	+++

To each 10 c c of medium NaOH or HCl was added in order to obtain the desired reaction. Two c c of 0.5M Na_2HPO_4 and 0.5M KH_2PO_4 mixtures (Na_2HPO_4 being alkaline and KH_2PO_4 being acid in reaction) were added in order to keep the H-ion concentration constant during the course of the experiment. By combining these solutions in certain proportions a series of tubes varying in reaction from $P_H + 4.3$ to $P_H + 9.0$ were obtained. Such a series is shown in table 1.

Three c c quantities were tubed and inoculated with 0.1 c c of a 1 : 10,000 dilution of a 24-hour "salt-free broth" culture. Growth was determined after 24 hours' incubation by the plate method. Triplicate plates were poured with veal infusion agar, $P_H + 6.8$-7.2, and counted after 24 hours. The remainder of the culture was sterilized in the Arnold for 30 minutes and the H-ion concentration determined. A control tube was also incubated and heated in the same way and the H-ion concentration determined. Variations in the H-ion concentrations were seldom found, except on the extreme alkaline side. These results are in accord with those obtained by Dernby and Avery[10], in their work on the growth of pneumoccoccus.

Isolation of Strains.—The strains were isolated on brilliant green mediums and transferred to peptic digest agar slants. For the experiments the cultures were purified on brilliant green or plain agar plates. Single colonies were transferred to veal infusion agar slants ($P_H + 6.8$-7.2) and kept at room temperature.

TABLE 4

OPTIMUM GROWTH OF B. TYPHOSUS; STOCK CULTURES

Blair 2*			Jacobs†			Number 1‡			Kearney§		
P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c
4.3	4.3	2,000				4.6	4.5	2,000	4.3	4.3	
4.8	4.8	97,000	4.8	4.8	0	4.9	4.8	2,000	4.8	4.8	<10,000
5.0	5.0	41,000,000	5.1	5.1	21,600,000	6.6	6.4	423,000,000	5.2	5.2	92,500,000
6.4	6.4	480,000,000	6.6	6.4	383,000,000	6.6	6.6	645,000,000	5.4	5.4	185,000,000
6.6	6.6	580,000,000	6.8	6.6	503,000,000	6.8	6.8	606.000,000	5.6	5.6	225,000,000
6.8	6.8	876,000,000	7.0	6.8	480.000,000	7.0	7.0	650,000,000	6.0	6.0	900,000,000
7.0	7.0	400,000,000	7.4	7.0	260,000,000	7.2	7.2	525,000,000	6.6	6.4	1,500,000,000
7.4	7.4	236,000,000	8.3	7.9	216,000,000	7.4	7.4	482,000,000	6.8	6.8	1,500,000,000
8.4	8.4	143,000,000	8.9	8.9	<1,000,000	8.3	8.6	320,000,000	7.0	7.0	2,500,000,000
9.0	8.8	8,250				8.9	8.9	200,000	7.4	7.2	1.810,000,000
									7.6	7.4	1,400,000,000
									8.2	8.0	171,000,000
									8.4	8.3	3,000,000
									8.6	8.6	<100,000

* Isolated from the blood in a severe case of typhoid complicated with cholecystitis and gall stones.
† Isolated from blood of a fatal case of typhoid, November, 1917.
‡ Isolated from a renal carrier, April, 1917.
§ Typical typhoid isolated from blood of a moderately severe case of typhoid, July, 1916.

Experimental Data.—The Influence of Salts on the Growth of B. typhosus: Preliminary experiments were conducted to determine the effect of varying concentrations of KCl, NaCl, KH_2PO_4 and Na_2HPO_4 on B. typhosus. The results of these experiments are shown in tables 2 and 3.

According to table 3 a 0.4 M solution of Na_2HPO_4-KH_2PO_4 mixture, is bacteriostatic, although it does prevent growth; a 0.3 M solution of phosphate is, however, more inhibitive than a 0.3 M solution of NaCl or KCl, etc. Since Na_2HPO_4 and KH_2PO_4 dissociate into 3 ions as compared to the 2 ions of the NaCl or KCl solution, a molal solution of Na_2HPO_4 or KH_2PO_4 has a greater osmotic pressure than a molal solution of KCl or NaCl. The ratio of the comparative strength of the solution to the osmotic pressure is about as follows: Molal concentration of NaCl : molal concentration of $Na_2HPO_4 = 2:3$ Isosmotic solutions of NaCl and Na_2HPO_4, therefore, produce about the same effect on B. typhosus. The phosphate concentration used in these experiments was kept far below that concentration which causes inhibition.

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[10] Jour. Exper. Med., 1918, 28, p. 345.

OPTIMUM GROWTH OF B. TYPHOSUS AND B. PARATYPHOSUS A. AND B. IN THE STANDARD "BUFFERED" MEDIUM

The determination of the optimum H-ion concentration for the growth of B. typhosus was conducted as previously stated. The results are shown in the following tables:

TABLE 5

OPTIMUM GROWTH OF B. TYPHOSUS; STOCK CULTURES

Strain No. 3*			Strain No. 5†			Strain No. 7‡		
P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c
4.6	4.6	130	4.6	4.6	100	4.6	4.6	100
5.0	5.0	99,000	4.9	4.9	19,000	5.0	5.0	18,700
6.0	6.0	384,000,000	6.0	5.9	323,000,000	6.6	6.5	836,000,000
6.5	6.4	1,163,000,000	6.4	6.3	583,000,000	6.8	6.8	1,605,000,000
6.6	6.4	1,473,000,000	6.6	6.6	600,000,000	7.0	7.0	1,326,000,000
6.8	6.7	1,540,000,000	6.8	6.8	773,000,000	7.4	7.4	810,000,000
7.0	7.0	2,130,000,000	7.0	7.0	630,000,000	8.7	8.7	50,500,000
7.4	7.4	810,000,000	7.4	7.4	570,000,000			
7.8	7.8	530,000,000	8.6	8.1	353,000,000			
8.7	8.7	50,500,000						

* Isolated from urine of patient suffering from renal calculi, November, 1916.

† Isolated from blood on the twelfth day of the disease, in a moderately severe case of typhoid, June, 1916.

‡ Isolated from blood in a moderately severe case of typhoid, October, 1916.

It will be seen from tables 4 to 7, inclusive, that the range of growth of B. typhosus is large. The optimum growth is between $P_H + 6.8\text{-}7.0$, or slightly on the acid side. Slight variations may occur, but most of the strains tested showed the same optimum range of growth. B. coli (Shohl and Janney), as compared to B. typhosus, shows a more pronounced optimum zone of growth and a large range. The results of 17 experiments on stock cultures have been averaged and are shown in the chart by the unbroken line. The logarithms of the number of organisms per cubic centimeter of medium are plotted as ordinates against the H-ion concentrations as abscissae.

TABLE 6

OPTIMUM GROWTH OF B. TYPHOSUS; RECENTLY ISOLATED CULTURES

Eldridge*			Eldridge Strain†			Lung Strain‡			M. Strain§			Joe Strain¶			"C" Strain		
P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c
5.8	5.8	150,000,000	6.0	6.0	300,000,000	5.8	5.8	500,000,000	5.9	5.9	190,000,000	5.6	5.6	254,000,000	6.0	5.9	280,000,000
6.4	6.4	250,000,000	6.4	6.4	350,000,000	6.4	6.4	940,000,000	6.4	6.4	550,000,000	6.0	6.0	526,000,000	6.2	6.0	270,000,000
6.6	6.6	310,000,000	6.6	6.6	465,000,000	6.6	6.6	1,120,000,000	6.6	6.6	570,000,000	6.4	6.4	690,000,000	6.6	6.6	449,000,000
6.8	6.8	460,000,000	6.8	6.8	480,000,000	6.8	6.8	1,010,000,000	6.8	6.8	530,000,000	6.6	6.6	726,000,000	6.8	6.8	410,000,000
7.0	7.0	480,000,000	7.0	7.0	405,000,000	7.0	7.0	960,000,000	7.0	7.0	540,000,000	6.8	6.8	886,000,000	7.0	7.0	392,000,000
7.2	7.2	410,000,000	7.2	7.2	445,000,000	7.2	7.2	1,010,000,000	7.2	7.2	510,000,000	7.2	7.2	856,000,000	7.6	7.4	456,000,000.
7.7	7.7	350,000,000	7.4	7.4	440,000,000	7.6	7.6	710,000,000	7.6	7.6	370,000,000	7.4	7.4	826,000,000	8.0	7.9	386,000,000
8.6	8.1	260,000,000	7.6	7.6	425,000,000							7.8	7.8	640,000,000	8.6	8.5	239,000,000

* Eldridge isolated from blood of a moderately severe case of typhoid, February, 1919.
† Eldridge isolated from stool of same patient. Optimum H-ion concentration determined immediately after isolation.
‡ Isolated from a case of influenza followed by a moderately severe case of typhoid. B. typhosus isolated from sputum.
§ Isolated from blood in a moderately severe case of typhoid, February, 1919.
¶ Isolated from blood in a mild case of typhoid in a vaccinated person, February, 1919.

TABLE 7

EFFECT OF VARYING H-ION CONCENTRATIONS ON GROWTH OF PARATYPHOSUS A AND B

Para A 11* Original Culture			Para A 11† Bile Strain			Para A 11‡ Duodenal Strain			Para A 8§			Para B 3¶		
P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c	P_H Cont.	P_H 24-Hour Culture	Growth per C c
4.3	4.3	100	4.3	4.8	33,500	4.3	4.3	300	6.0	6.0	703,000,000	6.0	6.2	740,000,000
4.8	4.8	2,000	5.2	5.1	28,200,000	4.8	4.8	2,500,000	6.4	6.4	746,000,000	6.4	6.4	910,000,000
5.0	5.0	16,300,000	6.4	6.4	636,000,000	5.2	5.1	11,000,000	6.6	6.6	801,000,000	6.6	6.6	960,000,000
6.6	6.6	460,000,000	6.6	6.5	1,003,000,000	6.4	6.4	425,000,000	6.8	6.8	1,266,000,000	6.8	6.8	1,190,000,000
6.8	6.8	513,000,000	6.8	6.8	553,000,000	6.8	6.6	543,000,000	7.0	7.0	1,136,000,000	7.0	7.0	1,410,000,000
7.0	7.0	683,000,000	7.2	7.1	690,000,000	7.2	7.0	543,000,000	7.2	7.2	796,000,000	7.2	7.2	980,000 000
7.5	7.5	708,000,000	7.5	7.4	626,000,000	7.5	7.4	556,000,000	7.4	7.4	896,000,000	7.6	7.6	990,000 000
7.8	7.8	556,000,000	8.6	8.2	386,000,000	8.4	8.0	216,000,000	7.8	7.8	393,000,000	7.8	7.8	836,000,000
8.3	8.2	450,000,000	8.8	8.6	453,000,006	8.8	8.6	184,000,000						
8.8	8.6	105,600,000												
8.9	8.9	>50,000,000#												

* Received from Captain Nichols. Isolated from Private Fraser in Colorna, Dublan, Mexico, August, 1916.
† Same strain taken from gallbladder carrier rabbit 816 days after infection.
‡ Same strain taken from duodenum of same rabbit.
§ Received from Captain Nichols, March, 1916, isolated from a blood culture.
¶ Received from the department of bacteriology of the University of Bern, December, 1913.
# Colonies too numerous to count.

It is evident that the rise in the curve on the acid side is greater than on the alkaline side. In other words, even though B. typhosus grows best in a slightly acid environment, it may be slightly more tolerant to alkalies than to acids.

One interesting point brought out by these experiments was that slight changes, near the limiting H-ion concentration, produced more marked changes in growth than approximately the same changes near the optimum H-ion concentration. For instance, in the "Blair" strain (table 4), a change from $P_H + 4.8$-5.0 caused a change in growth from 97,000 organisms per c c to 41,000,000 organisms per c c; while a change from $P_H + 6.4$-$P_H + 6.6$ resulted in a change of 10,000,000 organisms from 480,000,000-580,000,000. The "Kearney" strain failed to grow at at $P_H + 4.3$ and at 4.8, while at $P_H + 5.2$, 92,500,000 organisms were

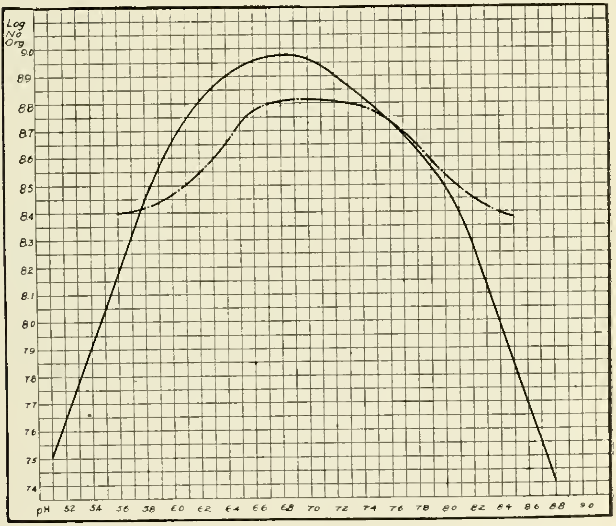

Chart 1.—Growth curve of B. typhosus. The growth of stock strains is represented by the unbroken line; the growth of recently isolated strains, by the dash and dot line.

counted per c.c. A change from 6.4 to 6.8 produced no recordable change in growth.

Another important observation was made. Recently isolated cultures had a decided optimal zone of growth which was more marked than in the stock cultures. The dash and dot line in the chart represents the results obtained by averaging 6 experiments conducted with recently isolated strains. Stock cultures exhibited an optimum zone with a definite optimum point of growth, while recently isolated strains developed in an optimum zone. Moreover, the range of growth of the latter strains was greater and the tolerance for alkali was more marked. This was particularly striking with the "C" strain, table 6. The growth of this strain was as profuse at $P_H + 7.9$ as at $P_H + 7.0$. The inhibition at $P_H + 8.6$ was not as marked as in the stock cultures. These observations were suggestive of bacterial adaptation to alkali and acid, and were considered of utmost importance in connection with the problem of urinary and gallbladder carriers.

These findings have some bearing on the selection of the medium to be employed in the isolation of B. typhosus. The optimum reaction for Endo-medium is $P_H + 7.8$-$P_H + 8.4$. Such a concentration of alkali is inhibitive for B. typhosus. The optimum reaction for a brilliant green medium is $P_H + 7.0$. As far as reaction is concerned, the latter medium is therefore preferable to Endo's agar. It was pointed out by Stickel and Meyer [1] that a negative finding of a stool containing few typhoid bacilli may be misleading when the usual Endo-medium is used. The results obtained in the present study fully confirm these views.

Table 7 shows the effect of varying H-ion concentrations on the growth of paratyphosus A and paratyphosus B.

It is obvious from table 7 that the paratyphoid strains showed an optimum growth at the same H-ion concentration as the typhoid strains, but exhibited a marked plateau on either side of the optium. The para B strains investigated are more alkali-tolerant than the para A strains.

Para A 2 was isolated from a rabbit which was sacrificed on the 816th day as a chronic gallbladder carrier. The bile and the duodenal strain, being recently isolated, were expected to grow in a plateau curve, but in our tests the reverse was noted, namely, the bile strain showed a decided optimum at $P_H + 6.5$ while the duodenal strain grew in a plateau from $P_H + 6.6$ to $P_H + 7.4$. The original stock culture behaved in a similar manner, namely, a plateau from $P_H + 6.6$-$P_H + 7.5$. In fact, the growth at $P_H + 7.5$ was slightly greater than at $P_H + 6.8$. These differences, which were determined by repeated tests, cannot be explained. It is not unlikely that the prolonged sojourn in a blocked gallbladder, the exposure to various products of inflammation, produced a tolerance to acid. This observation supports the conception that bacteria may undergo a process of adaptation while resident in the tissues of an immune host. Changes in the growth curve may be one of the newly acquired properties of the protoplasm which can be readily recorded. Growth curve studies may therefore be of value in the study of bacterial adaptation.

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[1] Jour. Infect. Dis., 1918, 23, p. 48.

SUMMARY AND CONCLUSIONS

B. typhosus has a range of growth from $P_H + 5.0$ to $P_H + 8.6$ with an optimum growth at $P_H + 6.8$-$P_H + 7.0$ in a salt-free veal infusion broth. Above or below these limits the resulting growth in comparison is very slight.

Large variations in the H-ion concentration near the optimum zone produce only slight effects on the growth of the organisms, while slight variations at the limiting zone produce a marked effect. These observations are fully in accord with the results reported by Cohen and Clark[12] in their studies on the growth of certain intestinal organisms at different concentrations. In the region near the optimum H-ion concentration the tolerance for alkali seems to be slightly greater than for acid.

Stock cultures isolated from stools, blood and urine of typhoid patients or carriers have a more decided optimum than recently isolated cultures of similar cases. In such cultures the plateau of the growth curve is much more pronounced and extends over a wider range than in stock cultures. The latter is suggestive of microbic adaptation to changes in H-ion concentration in body fluids, particularly urine and bile.

B. paratyphosus A and B have a range of growth at varying H-ion concentrations similar to that of B. typhosus but exhibit a greater tolerance for alkali than B. typhosus.

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[12] Jour. Bacteriol., 1919, 4, p. 409.

DO "CARRIER" STRAINS DIFFER FROM STRAINS ISOLATED FROM ORDINARY TYPHOID CASES ?

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. III

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This question involves the consideration of several factors which may play a part in the successful production of a high percentage of experimental gallbladder or renal carriers. It should be emphasized that this study was primarily undertaken to elucidate certain facts recorded in the literature pertaining to animal carriers. It was not the intention to attempt to prove or to refute the conception that the typhoid strains isolated from human carriers may differ in their invasive or infective properties, a phase of the problem which is practically impossible when one considers that the experimental reproduction of typhoid fever in laboratory animals cannot be accomplished. The customary method of testing the virulence of typhoid strains by intraperitoneal injections into guinea-pigs does not reproduce the conditions as they operate in human infections and the data collected by this method cannot be used for comparison. Neither Lentz [1] nor Ledingham [2] noted by the use of this procedure differences between the virulence of the carrier strains and those isolated from ordinary typhoid cases. Some incomplete observations of Ledingham, however, suggest that the virulence of the typhoid strains isolated from one and the same carrier may vary for guinea-pigs at different times of the year. For example, a strain isolated from an intestinal carrier exhibited a reduced virulence during the winter months. Using a similar method of testing for virulence, Remlinger [3] noted that typhoid bacilli found in liver abscesses were avirulent, and Niepratschk [4] isolated from a renal carrier a strain of low virulence. Furthermore, Levy and Gaehtgens [5] recorded by means of bacteriotropin tests the observations

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* Dr. C. R. Christiansen, who died from influenza in October, 1917, conducted during the years 1915-16 the majority of the experiments to be reported. This paper should stand as a memorial to his enthusiastic and resourceful collaboration in our typhoid studies.

[1] Klin. Jahrb., 1905, 14, p. 475.

[2] The Carrier Problem in Infectious Diseases, London, 1912, p. 110.

[3] Compt. rend. Soc. de biol., 1897, 4, p. 110.

[4] Ztschr. f. Hyg. u. Infektionskr., 1909, 64, p. 454.

[5] Ergebn. d. allg. Path. u. path. Anat., 1915, 18, p. 491.

that the virulence of the typhoid bacilli excreted by typhoid patients was as a rule greater than the one of carrier strains. It has been suggested by Hilgermann [6] that the relative infrequency of infections in the entourage of carriers may be due to variations in the virulence of the organism and that this diminishes with the continued parasitic existence of the latter in the organs of its host. Based on some observations on rabbits Wagner and Emmerich [7] came to the same conclusions. The well-known studies by Metchnikoff and Besredka [8] on experimental typhoid fever demonstrated that typhoid bacilli shed by a chronic carrier are pathogenic for small laboratory animals, but that they are unable to transmit typhoid to chimpanzees. The general notion that carrier strains are less virulent than those isolated from acute typhoid cases finds, therefore, some justification, and the question of the occurrence of saprophytic and parasitic strains is again placed in the foreground. Closely interwoven with the answer to the original question is the consideration of the possibility that carrier strains may possess specific elective properties for the tissues from which they are isolated. Ledingham,[9] in discussing this subject, expresses the opinion that this hypothesis can be proved. A carrier strain that has sojourned for long periods in man may conceivably be more highly endowed with those properties which permit of its continued vegetation within the body after the infection is past than a typhoid strain responsible for a water-borne epidemic, which gets little opportunity of becoming accommodated to the human organism. As far as we know, the conception of elective organotropism in the sense of Rosenow [10] has not been considered experimentally for the typhoid bacillus but has been fully proved by Fränkel and Much [11] for a paratyphoid B bacillus obtained from a case of purulent cholecytitis.

Early in the course of our typhoid work, we felt the necessity of studying these problems pertaining to the inherent properties of the typhoid bacillus before we undertook a systematic study of the factors responsible for the occurrence of gallbladder, liver or renal carriers. We were fortunate in having access to carrier strains, which were responsible for extensive and serious epidemics. The dynamic properties of some of these strains as far as they concern man had been well investigated epidemiologically. These investigations were at our disposal through the publications and verbal informations obtained from Drs. W. A. Sawyer and F. G. Cummings. Additional cultures were isolated from carrier cases in the wards of the University of California Hospital and compared with recently isolated strains of our own and those we received through the courtesy of Drs. H. T. Chickering, J. V. Cooke, and the Bureau of Communicable Diseases of the California State Board of Health. The available carrier strains were not very numerous, but could at least be considered representative of the variety of typhoid bacilli as they occurred in a large community.

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[6] Klin. Jahrb., 1908, 19, p. 463.

[7] Med. Klin., 1916, 12, p. 819.

[8] Ann. d. l'Inst. Pasteur, 1911, 25, p. 193.

[9] The Carrier Problem in Infectious Diseases, pp. 75 and 76.

[10] Jour. Infect. Dis., 1916, 14, p. 527.

[11] Ztschr. f. Hyg. u. Infektionskrankh., 1911, 69, p. 342.

The analysis of these typhoid strains covered originally two questions: First, are there any cultural or biochemical differences between carrier strains and those recently isolated from acute typhoid cases? Second, do carrier strains exhibit a specific elective behavior for certain tissues in the rabbit, and can this elective localization be acquired by successive passage through the gallbladder of this species of animal? A study of carrier strains isolated at different time intervals from one and the same case was contemplated, but the specimens necessary for this investigation could not be procured with desirable regularity.

In the course of the analysis of the preliminary data, it was realized that an additional question could be advantageously investigated on the same animals, namely, Do immunized rabbits develop a gallbladder carrier state more readily than normal animals? This question was first raised by Fornet [12] in connection with his theory that the carrier state is to be regarded as a consequence of established partial immunity to the typhoid bacillus, and that, in fact, the bacilli discharged by the carrier may not necessarily be descendants of those that caused the primary infection. A reinfection takes place, which, according to his views, is not accompanied by any clinical symptoms. The region attacked by the bacilli in this reinvasion would have acquired, in consequence of the primary infection, such a tolerance to the micro-organisms as to render its saprophytic existence possible. Fornet mentioned, further, in his first article that animal experiments to support his contention were in progress. According to a more recent review,[13] he apparently gained from a few preliminary experiments the impression that immunized rabbits developed more readily and more regularly a typhoid carrier state by alimentary infection than nonimmunized

[12] Ztschr. f. Hyg. u. Infektionskrankh., 1909, 64, p. 365.

[13] Ergebn. d. innern Med. u. Kinderheilk., 1913, 11, p. 205.

animals. In the same article,[14] he suggested that similar results may be obtained in animals infected by the intravenous route. We will have occasion to discuss, in a paper dealing with the feeding of typhoid and paratyphoid bacilli to rabbits, the impossibility of producing a gallbladder infection by the alimentary method. This phase of Fornet's theory is unquestionably incorrect. One may recall in this connection that the carrier state supposed to have been produced by the inoculation of one half of a standard 10% blood-agar culture of a certain strain of B. typhosus, has been recommended as a means for testing the immunizing power of a given typhoid vaccine. Nichols[15] and others were unable to repeat these results. The experiments of the latter are supported by our own observations, namely, that typhoid immunized rabbits develop a carrier state in a higher percentage of instances than normal animals. The occurrence of local infections in immunized or possibly sensitized animals has some bearing on the general problem of focal infections in man. Numerous observations reported in the literature indicate that immunization does not prevent the development of local infections in rabbits. One gains the impression that such procedures quite frequently favor it. In this connection the work of Faber[16] on streptococcic arthritis, and of Wadsworth[17] on pneumonia in rabbits, should be mentioned. Furthermore, Creig[18] reports the development of a gallbladder carrier state in rabbits progressively immunized with living cholera-like vibrios, while Flu[19] notes similar lesions in animals repeatedly infected with Flexner dysentery bacilli. The factors responsible for these results have been investigated. and will be discussed in this and in subsequent papers.

THE CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF "CARRIER" STRAINS

During the last five years 14 B. typhosus strains, isolated from carriers in California. were studied in detail by means of the usual cultural and carbohydrate tests. The histories of the strains used in the animal experiments are indicated in table 1. The epidemics caused by strains 10 and 21, are discussed by Sawyer[20] and the infections provoked by strain 4 have been reported by Cummings.[21] Strains 18, 49, 1 and 84 are stock strains, or were isolated by us from necropsy material, or from secretions of carriers under our control. These cultures, in addition to 7 others, were studied by the methods recently described, in connection with the analysis of 2 irregular typhoid strains.[22] No differences from the ordinary typhoid strains could be noted; the behavior on carbohydrate medium, milk and on rhamnose plates was typical. All the strains were rapid xylose fermenters and failed to attack dulcite in liquid mediums. These observations are quite in harmony with the findings of E. Müller,[23] who studied 19 carrier strains. According to his results, the strains of B. typhosus obtained from intestinal carriers showed no abnormalities, nor did the cultures exhibit any indication of newly acquired properties.

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[14] Ibid., p. 216.
[15] Jour. Exper. Med., 1914, 20, p. 573.
[16] Jour. Exper. Med., 1915, 22, p. 615
[17] Am. Jour. Med. Sc., 1904, 128, p. 851.
[18] Indian Jour. Med. Research, 1915-16, 3, pp. 259 and 397.
[19] Geneesk. Tidjschr. v. Nederl. Indie, 1918, 58, p. 67.
[20] Jour. Am. Med Assn., 1912, 58, p. 1336; 1914, 63, p. 1537

All strains were specifically agglutinated by a polyvalent typhoid immune serum. The variation of the individual strains differed in no respect from those commonly encountered by Bull and Pritchett [24] with the ordinary typhoid strains. As most of our tests were made previous to the findings of Hooker,[25] who clearly demonstrated consistent antigenic differences among some strains of B. typhosus, absorption tests were not done with the freshly isolated strains, and our inquiry along these lines is therefore incomplete. Recently a few tests conducted with the strains cultivated on agar for from 2-7 years showed that strains 10, 4 and 84 belonged to group 2 and strains 18 to group 3 of Hooker's classification, while strains 21, 49 and 1 exhibit the characteristics of group 1. Hooker had already found, in his studies in 1916, that our strain 18 and his strain 4 fell in group 3. It is not unlikely that such tests would have produced different results had they been carried out when the strains were originally isolated. The existence of atypical and irregular typhoid strains in carriers should be seriously considered in the light of our recent observations. Moreover, the publication of Downs [26] suggests the presence of antigenic differences among typhoid strains and a subdivision into at least 5 groups. Confirmation of these observations as to the occurrence of various types of typhoid bacilli would be of considerable epidemiologic importance in the analysis of "carrier" borne typhoid outbreaks, and may perhaps explain the variable infectivity of carriers. Gruber,[27] for example, was able to trace a carrier through the peculiar behavior in glycerol of the causative typhoid strain, the so-called B. metatyphosus of Mandelbaum. An intensive study of carrier strains would perhaps also explain the findings of Schlemmer,[28] who noted that one and the same carrier may eliminate at varying time intervals strains of typhoid bacilli, which are nonsusceptible to the bactericidal properties of the same immune serums. These observations involuntarily lead to a consideration of the virulence of our carrier strains. There is no experimental animal on which one can successfully test the relative virulence of typhoid strains, but to satisfy our curiosity we applied the method of Ledingham [29] to strains 1, 49 and 84. These organisms killed guinea-pigs weighing 250 gm. in quantities of not less than 1 c c of a 24-hour old broth culture. Strain 49 killed in 2.5 c c amounts. In comparison with the ordinary typhoid strains 40, 41, 54, 55 and 56, which were fatal to guinea-pigs in 1-2 c c amounts, the virulence of the 3 carrier strains cannot be considered exceptionally low.

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[21] Jour. Am. Med. Assn., 1917, 68, p. 1163.

[22] Meyer, K. F., and Neilson, N. M.: Jour. Infect. Dis., 1920, 27, p. 46.

[23] Centralbl. f. Bakteriol. I. 1909, 53, p. 209.

[24] Jour. Exper. Med., 1916, 24, p. 35.

[25] Jour. Immunol., 1917, 2, p. 1.

[26] Abstr. Bacteriol., 1920, 4, No. 56, p. 19.

Our findings on carrier strains differ therefore in one respect from those reported by Lentz, Ledingham, E. Müller and others, and justify the definite and final conclusion that culturally and biochemically carrier strains do not differ in any way from those recently isolated.

THE BEHAVIOR IN THE RABBIT OF CARRIER STRAINS AND OF STRAINS RECENTLY ISOLATED FROM ACUTE INFECTIONS.

In accordance with the problems discussed in the introduction, we tested the theory of Fornet and investigated the conception of elective organotropism advanced by Rosenow and Brown,[30] using a number of carrier strains at our disposal. These tests were paralleled by similar experiments with recently isolated strains and with those artificially cultivated for several years. Such a comparison was necessary in order to prove the following points: (a) the validity of the conclusions drawn from the experiments conducted with carrier strains; (b) the observations of Weinfurter,[31] Nichols,[15] and others which indicated that freshly isolated strains produced gallbladder lesions in a higher percentage of instances than old strains; and (c) the assumption that a "certain" strain of B. typhosus cultivated on blood agar is necessary to provoke a carrier state in rabbits. Furthermore, our ulterior motive in conducting these tests centered on the desire to find a typhoid strain which was particularly suited for carrier studies on rabbits.

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[27] Arch. f. Hyg., 1913, 80, p. 272.

[28] Ztschr. f. Immunitätsforsch. u. exper. Therapie, 1911, 9, p. 149.

[29] The Carrier Problem in Infectious Diseases, pp. 109 and 110.

[30] Arch. Int. Med., 1919, 23, p. 185.

[31] Centralbl. f. Bakteriol., I. O., 1915, 75, p. 379.

Methods.—The rabbits used in this series of experiments weighed between 2,000 and 3,000 gm., and were supplied from the same source. During the years 1915, 1916, and 1917 these animals were free from coccidiosis and spontaneous cholecystitis. Little attention has been paid to this infection by the majority of workers on experimental typhoid, and sweeping conclusions on gallbladder infections have been drawn from the results obtained on rabbits, which possessed, according to the published protocols, livers heavily infested with coccidiosis. Some investigators (Gotschlich[32]) are aware of the fact that coccidiosis of the liver favors the localization of bacteria in the biliary passages and the gallbladder, while others intentionally or unintentionally ignore this infection. Experimental conclusions on cholecystitis appear justified only when one is certain that the animal does not possess predisposing infections. Many statements relative to the value of a method for the production of carriers, expressed in percentages of infections, are seriously invalidated by the utter neglect of these fundamental requirements.

The animals were kept on a mixed oat, hay and cabbage diet, in single "Lewis" cages and in a fly proof isolation room. The sawdust bedding was disinfected by lysol or strong lye solutions.

Immunization was accomplished by at least 6 intravenous inoculations, at 6-7 day intervals, of heat killed (53-54 C.) (McÇoy), tricresolized, autogenous cultures grown on rabbit-blood agar. The vaccine amounts were increased progressively from 1/20 to 1/2 of a slant. Ten to 15 days after the last injection of the vaccine, the animals were infected with the same strain of typhoid bacilli in a living state. For these tests the cultures were purified on Endo plates, and kept on veal agar or grown on rabbit-blood agar slants for at least 5 generations before use. The preparation of the mediums, the selection of the tubes and proper slanting were conducted in accordance with the specifications ordinarily employed. We intend to discuss in another paper of this series the observations made in connection with the cultivation of typhoid strains on rabbit-blood agar, but attention is now called to the fact that in our experience never more than 40-60,000 million typhoid bacilli grew on the specified agar surface. The figures determined by Gay and Claypole and faithfully copied by Stone,[33] namely, 1,400,000 million organisms, are probably incorrect. In order to reduce the initial mortality caused by the inoculation of large doses of living typhoid bacilli into rabbits, we determined when possible the minimum lethal dose, and found that from 2-5 billions per kilogram of rabbit weight are fairly well borne by normal animals weighing over 2,000 gm. Expressed in terms pertaining to blood-agar slants, these amounts varied from 1/10 to 1/4 slant per rabbit. All inoculations were made with unstrained suspensions in sterile saline solution using a marginal vein, and in one series of animals the renal artery.

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[32] Handb. d. path. Mikroorganism, Ed. 2, 1912, 1, p. 217.

[33] Jour. Infect. Dis., 1919, 25, p. 290.

The rabbits were killed in from 20-35 days after the inoculation. Cultures were made on Endo-agar plates or by enrichment of portions of the organs in 10% ox bile rabbit broth. The major portion of the data presented was collected early in the course of our study on experimental typhoid carriers. The conception of the carrier state at this period was comparatively simple, namely, the presence of typhoid bacilli in the bile and gallbladder wall constituted a positive result.

The data presented in tables 1 and 2 should be considered from this point of view. An animal was noted as "died" when it succumbed to the intoxication in the first 24-48 hours.

TABLE 1

Gallbladder Carriers Produced in Rabbits by the Injection of Old and Recent "Carrier" Strains of B.Typhosus

Strain	Age of Strain	No. of Animals		Positive		Negative	
		Immune	Normal	Immune	Normal	Immune	Normal
Stool and Osteomyelitis:							
10 "O" responsible for 26 contact cases in 3½ years	3½ years	5	5	1	1	4	4 (died)
18 "Cr" tibial abscess 2 years after an attack of typhoid fever	3 years	4	2	1	0	3	2
21 "L" responsible for 93 cases in one epidemic	1 month	6	8	5	5	1	3 (died)
4 "H" responsible for 23 cases in a food epidemic	2 months	3	—	3	—	—	—
49 gallstone, negative, no history of typhoid fever	3 days	3	3	0	1	3	2
Urine:							
"1" renal carrier, injection made through the left renal artery	3-4 months	6	2	6	2	0	0*
84 "renal carrier," right typhoid pyelonephritis	Immediately urine sediment of 200 c c of urine 10 billion organisms	2	2	1	2	1	0*
7 strains................	Immediately to 3½ years	29	22	17 56%	11 50%	12 44%	19 50% 9% died

* Kidneys of all animals negative.

Experimental Data.—The various experiments are shown in concrete form in tables 1 and 2. It is quite apparent that with the exception of strains 21 and 1, none of the carrier strains possessed elective properties for the gallbladder of rabbits. The percentage of infections in 51 rabbits inoculated with carrier strains was lower than that found in 70 rabbits similarly infected with recently isolated strains of B. typhosus. The highest percentage of infections was noted with a freshly isolated strain 21 which was introduced intravenously, and with a 3-4 month old culture (1) which was injected through the left renal artery. The latter experiment will be discussed elsewhere more in detail, but in connection with it attention should be called to the fact that operative technic, fasting of the animals with the resulting stasis in the gallbladder, unquestionably contributed to the permanency of the infection in the biliary passages. Furthermore, this experiment with strain 1, as well as that conducted with 84, lend little support to the contention that the parasitic typhoid strains isolated from chronic renal carriers possess for the rabbit at least elective organotropic properties.

TABLE 2

GALLBLADDER OR LIVER CARRIERS PRODUCED IN RABBITS BY THE INJECTION OF RECENT AND OLD STRAINS OF B. TYPHOSUS ISOLATED FROM STOOL BLOOD CULTURES

Strain	Age of Strain	No. of Animals		Positive		Negative	
		Immune	Normal	Immune	Normal	Immune	Normal
Recent Blood Cultures:							
24	28 days	10	12	8	5	2	7
26	55 days	2	2	1	2 (died)	1	0
27	6 months	1	1	1	1	0	0
28	5 months	1	2	0	0	1	2
29	5 months	2	2	1	1 (died)	1	1
36	53 days	1	2	0	1	1	1 (died)
37	2 months	1	1	1	0	0	1
38	1½ months	1	1	0	0	1	1
40	2½ months	1	1	0	1	1	0
41 (severe case)	3 months	1	1	1	1 (died)	0	0
45	3½ months	1	2	0	1 (died)	1	1
46	2 months	1	1	1	0	0	1 (died)
48	1 month	2	1	2	1	0	0
50	1 month	1	1	1	1	0	0
52*	2½ months	2	2	0	1	2	1 (died)
53*	2 months	1	1	1	1 (died)	0	0
54*	2 months	1	1	1	1	0	0
55*	1 month	1	1	1	1	0	0
56*	1 month	1	1	1	0	0	1
Chr. 2	4 months	1	1	1	1	0	0
Total		33	37	22	20	11	17
Average percentage of carriers				66%	54.3% (25% died)	33%	45% (17% died)
Old Stock Cultures:							
15 ("I")	3 years	2	0	1	0	1	0
17 (old)	>3½ years	1	—	0	—	1	—
14 (Hopkins)	>4 years	2	2	0	0	2	2
6 (Rawlings)	15 years	9	5	2	0	7	5
23 (Dorset)	16 years	2	2	1	1	1	1
Total		16	9	4	1	12	8
Average percentage of carriers				25%	11%	75%	88.8%

Quite recently Lentz, Hailer and Wolf [34] reported similar tests with eight carrier strains. Each culture was inoculated in one loopful amounts into 5 rabbits. Only 30% of the animals developed a carrier state. These tests were undertaken to prove whether or not the strains employed by Doerr [35] and by Johnston [36] possessed specific elective properties, which could explain the high percentage of infected gallbladders recorded by these writers. It is evident that our experiments confirming those of Lentz [34] fail to support the conception of elective parasitism.

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[34] Arb. a. d. Reichs Gsndhsamte, 1918, 51, p. 1.

Our observations support the findings of Weinfurter [31] and of Nichols,[15] namely, that recently isolated parasitic strains produce a higher percentage of carriers than old saprophytic strains. But it is also demonstrated that even such strains failed to produce biliary infections regularly. The high initiated mortality of 17-25% among the nonimmunized rabbits must, however, be considered a serious and expensive disadvantage.

How do our results compare with those reported by 15 other workers? We have attempted to analyze their reports and have summarized the data in table 3. In this summary it is difficult to estimate the number of organisms inoculated, the weight and age of the rabbits, and the time period elapsing between infection and necropsy, facts which interfere seriously in estimating the true value of the experiments. Making allowance for these and other factors like coccidiosis, individual resistance, diet, etc., it is quite obvious that the average percentage of gallbladder carriers produced does not exceed 65%. This figure may be accepted for our analysis. A variety of strains was employed by the different experimenters. Old and recently isolated strains and even carrier strains were chosen by Morgan,[37] by Hailer and Rimpau,[38] by Hailer and Ungermann,[39] and by Lentz, Hailer and Wolf.[34] The evidence supports our conclusions that recently isolated strains, whether of carrier or acute typhoidal origin, produce gallbladder infections more frequently than old stock cultures. Carrier strains offer no advantage over recently isolated strains of acute typhoid cases. In our opinion the recognition of the fact that a large number of organisms is necessary to insure biliary infection is important. In this connection the high percentage of deaths should also be considered. Most of the workers in this field of experimental pathology fail to state the actual loss of animals in the 24 to 72 hours

[35] Centralbl. f. Bakteriol. I. O, 1905, 39, p. 624.
[36] Jour. Med. Research, 1917, 37, p. 189.
[37] Jour. Hygiene, 1911, 11, p. 202.
[38] Deutsch. med. Wchnschr., 1912, 38, p. 2267.
[39] Arb. a. d.Gsndhsamte, 1914, 47, p. 451.

following the injection of large doses of living typhoid bacilli. In our series we recorded as high as 25% mortality in nonimmunized animals, and we intend to publish several series of tests in which the mortality rose to 50%. Every worker will agree that such a method is prohibitive and wasteful. It will be the purpose of subsequent papers to discuss the procedures that were found of value in overcoming this loss. By these methods it was possible to raise the percentage of positive carriers to from 90-100% of the animals infected.

TABLE 3

PERCENTAGE OF GALLBLADDER CARRIERS PRODUCED BY VARIOUS WORKERS

Author	Weight of Rabbits	Number of Bacteria Injected		Percentage of Carriers
		As Stated by Author	Estimated by Us	
Forster and Kayser......	2.0 - 2.6	0.5–0.6 mg.	600–15,000 million	Not regular infected on 5th day
Doerr....................	1.13- 2.0	¼–2 loopful	600– 5,000 million	90% in 10 rabbits
Lemiérre and Abrami....	—	2 c c of Widal's agglutination fluid	1,000– 2,000 million?	Irregular after 6th day, recovery
Chiarolanza.............	2.0 - 2.5	½–4 loopful	1,250–10,000 million	74% in 23 rabbits
Blumenthal, E.	—	1 loopful	2,500 million per kg. = 5-9,000 million per animal	100% in 5 rabbits
Bully....................	1.16- 2.0	1 loopful per kg.	2,500 million per kg. 2,500-5,000 per animal	48% in 40 contral rabbits
Morgan.................	Aver. 2.52	4 c c of broth	2,800–3,200 million	50% in 10 rabbits
Johnston...............	0.98-1.94	0.5 c c of an agar suspension	?	?
Perussia................	—	1-4 loopful per kg.	2,500–10,000 million per kg.	46% carriers in 15 rabbits
Weinfurter..............	2.5	¼-3 loopful	600–7,500 million	53% in 58 rabbits
Gay and Claypole.......	2.0-2.5	½ standard blood agar slant 720,000 million (?)	20,000–25,000 million	93.0% in 28 rabbits 90.6% in 43 rabbits 74-76% in ? rabbits (Beckwith)
Cummins and Cumming	—	1/10 slant	2,500–4,000 million	71% in 7 normal and immune rabbits had liver infected; 29% in 7 rabbits had infected gallbladder
Nichols....	2.0-3.0	½ fatal dose = 1/10 slant of blood agar	4–5,000 million	21% in 16 rabbits, 28.8% in 45 rabbits, 40% in 10 immune rabbits
Hailer and Rimpau and Hailer and Ungermann	2.5-3.0	1 loopful per kg.	6,000–7,000 million per animal	75% in 61 rabbits killed 8-34 after injection
Lentz, Hailer and Wolf..	—	1 loopful	2-5,000 million	30% in 40 rabbits
Teague and McWilliams	1.4-2.4	Large dose 8,000–15,000 million	—	60% positive in 10 rabbits 3-24 days
Stone....................	1.7-4.3	⅓ blood-agar slant 460,000 million (?)	10–20,000 billion	92% (Beckwith states 94%) in ?
Beckwith................	—	⅓ blood-agar slant	10–20,000 billion	100% in ? rabbits

Analyzing the data in tables 1 and 2, we found to our surprise that immunized rabbits became carriers more readily than nonimmunized animals, an observation which confirmed the theory of Fornet, to which we had had no access at the time our deductions were originally drawn. Nichols [15] also noted a higher percentage of infection among his immunized rabbits, as did Cummins and Cumming.[40] Certain requirements must be fulfilled to obtain these results. It was found that immunized rabbits eliminated a larger number of typhoid bacilli than normal animals when the test inoculation was made on the 6-12th day after the last injection of the vaccine. Additional factors unquestionably play an important rôle, but it is not the purpose of this paper to discuss them. So much can, however, be said: The evidence at our disposal does not permit us to conclude that the results obtained on immunized rabbits are applicable to man and to the human carrier state.

Furthermore, it is evident that the development of the carrier state in rabbits cannot be used as a test for the potency of vaccines. Our rabbits were protected by 6 to 7 inoculations of a fresh vaccine prepared from the strain which was used for the test inoculations. Moreover, the intravenous doses were not larger than those usually employed. It has been argued that Nichols obtained his gallbladder infections in immunized rabbits because the intravenous test dose of one half fatal dose = 1/10 slant = 4-5 billion organisms was sufficient to overcome the increased bactericidal effect of the blood (which, by the way, does not exist). If this argument is sound, the same conclusion must apply to those studies, in which about 720 billion (?) living B. typhosus were employed and in which the reverse is reported as having occurred. In this connection one may state that even immunization with living bacteria does not alter the final outcome. Independent of the degree of protection, a certain percentage of rabbits that have recovered will develop the carrier state. This result is not surprising when one appreciates that, according to Parker and Franke,[41] whose studies we have confirmed by extensive experimentation, the destruction of intravenously inoculated typhoid bacilli progresses with the same speed in immunized as in normal rabbits. No difference can be recorded in the bactericidal power of normal and immunized rabbit tissues. The infection of the gallbladder and the persistence in it of viable bacteria depend on certain factors operative in the liver, its vascular system, the bile and the diet of the animal (coccidiosis excluded) and not on the bactericidal properties of the blood.

[40] Jour. Roy. Army Med. Corps, Lond., 1914, 22, p. 378.
[41] Jour. Med. Research, 1919, 39, p. 301.

CAN TYPHOID STRAINS ACQUIRE ELECTIVE CHOLECYSTOTROPIC PROPERTIES BY SUCCESSIVE PASSAGE THROUGH THE GALLBLADDER ?

The studies reported in this chapter were undertaken in 1915-16, when the influence of Rosenow's doctrines permeated the bacteriologic and medical literature. Elective localization had been induced with the plague bacillus, the staphylococcus, the streptococcus, the colon bacillus, the paratyphoid bacillus and perhaps the representatives of the Brucella group, but no reports on the typhoid bacillus were available. As we were searching in our carrier studies for an organism which regularly localized in the gallbladder, the contemplation and execution of a series of passage experiments had at that time some justification and interest. Today, after having analyzed the mechanism of this infection in the rabbit, our efforts of this period appear superfluous. The course of the experiment is shown in tabulated form in table 4. By direct inoculation into the gallbladder the recently isolated typhoid strain was perpetuated through 4 rabbits. Each successive infection was done by transference of the infected bile from the viscus of the preceding, laparotomized rabbit. The strain was lost in the third passage; 15 days after the inoculation into Rabbit *722* the gallbladder bile was found sterile. This observation proves in the first place the contention of Hailer and Ungermann [39] that even the method of intravesicular inoculation of typhoid bacilli is not absolutely dependable, and that persistence of the bacteria may be of comparatively short duration. Furthermore, this typhoid strain, after it had been resident in the gallbladder for 116 days, had not acquired any selective property for growth in this viscus. This conclusion is also supported by the tests conducted on 33 rabbits, which received intravenously the first or second generation of the bile culture on blood-agar slants. The percentage of carriers (45 and 22%) differed in no respect from that noted for the recently isolated, nonlapinized strains. One may, therefore, conclude that elective cholecystotropic properties cannot be conferred on typhoid strains by successive passage through rabbits. This conclusion has, during the past 4 years, been repeatedly confirmed by experiments with typhoid or paratyphoid strains, which had been resident in the gallbladder of the rabbit from 230 to 816 days. In not one instance did these carrier strains exhibit elective properties. Prolonged sojourn in the rabbit tissues produced changes in agglutinability, as has been observed by Lange and Roos [42] and by Wagner and Emmerich.[7] Nearly 50% of the gallbladder strains were temporarily inagglutinable. A paratyphoid A strain, which persisted for 816 days in the gallbladder and the liver, was hyperagglutinable and clumped spontaneously. Intravenous injections of 10,000 million bacteria of this strain (second generation) were well tolerated by 5 rabbits. At necropsy 10 to 25 days after the injection these animals exhibited normal gallbladders. The strain had undergone some fundamental changes, but had not acquired specific invasive properties. An explanation of the carrier state can therefore not be expected on the basis of an elective localization in the sense of Rosenow.

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[42] Arb. a. d. Kais. Gsndhtsamte. 1915, 50, p. 57.

TABLE 4

Passage of a Recently Isolated Strain of B. Typhosus (K) Through the Gallbladder of Several Rabbits

1st Passage. Rabbit 790 (2d generation intravesicularly)
14 days
Rabbit 723 intravenously 1st generation, positive
Rabbit 724 intravenously 1st generation, positive
Rabbit 725 intravenously 1st generation, negative
Rabbit 726 intravenously 1st generation, negative
Rabbit 791 intravenously 2d generation, positive (died)
Rabbit 792 intravenously 2d generation, positive
Rabbit 793 intravenously 2d generation, negative (died)
Rabbit 794 intravenously 2d generation, negative
Rabbit 795 intravenously 2d generation, negative (died)
Rabbit 796 intravenously 2d generation, negative
Rabbit 798 intravenously 2d generation, positive

2d Passage. Rabbit 797 (bile of 790 intravesicularly)
35 and 70 days, resp.
5th generation of 790 and 2d generation of 797
8 immune rabbits, all negative
14 normal rabbits, 5 died in 24 hours, positive
2 positive on necropsy
7 negative on necropsy

3d Passage. Rabbit 722 (bile of 797 intravesicularly)
Negative on 25th day

SUMMARY

A comparative study of 14 carrier strains failed to reveal any striking differences between these strains and those isolated from acute typhoid cases. Seven carrier strains tested on rabbits by means of intravenous injections failed to exhibit specific elective cholecysto- and renotropic properties. Furthermore, it was impossible to confer such characteristics to a recently isolated strain of B. typhosus by successive passage through the gallbladder of rabbits.

It was, however, demonstrated that immunized rabbits inoculated with large doses of living typhoid bacilli exhibited gallbladder infections in a somewhat higher percentage of instances than normal rabbits. The theory of Fornet and the publications of other workers on experimental typhoid carriers are discussed and compared with our own observations.

XXIII.

A COMPARATIVE STUDY OF THE INFECTIONS PRODUCED BY INTRAVENOUS INJECTIONS OF TYPHOID, PARATYPHOID A AND B BACILLI IN NORMAL AND IMMUNIZED RABBITS

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. IV

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In order to establish a "carrier state" with absolute regularity, it is unquestionably necessary to obtain as clear a picture as possible of the cycle of events which follows an intravenous injection of gram-negative bacilli in the rabbit. The results of a number of experiments, dealing with the fate and disappearance of the typhoid and paratyphoid bacilli traced from the blood through the various organs are presented in this paper. In subsequent publications of this series, the removal of typhoid bacilli from the blood, their appearance in the bile and urine, the behavior of the leukocytes, etc., will be treated separately. Observations recorded in paper 3 suggested an analysis of the distribution and disappearance of typhoid bacilli in the organs of immunized rabbits. This study was part of a more general analysis of the mechanism of typhoid immunity in the rabbit, guinea-pig, cat, rat and monkey, and was undertaken in the form of a survey to prove or to refute the rather plausible conception of a cellular immunity in typhoid fever.

Observations on guinea-pigs also suggested some experiments with paratyphoid A and B bacilli. It will be demonstrated that paratyphoid A behave like typhoid bacilli in normal and immunized rabbits, while the paratyphoid B bacilli are more rapidly destroyed in the immune than in the normal rabbit. The mechanism responsible for the resistance and the ability of the normal, immune or immunized rabbit to destroy a large number of bacteria of the typhoid-paratyphoid group has been

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investigated in a few preliminary experiments. But interwoven with all these detailed studies are the main questions: Why does the gall-bladder become infected? Why does this viscus quite frequently retain the infection? The experiments recorded in this paper contribute some facts to the solution of this problem.

The technical execution of the contemplated extensive experimental program progressed rather slowly on account of the war, but was practically completed, when the studies of Parker and Franke [1] and of Stone [2] appeared in print. The results reported by the latter are contrary to those reported by Parker and Franke; namely, Stone claims to have demonstrated that typhoid bacilli disappear "more quickly from the organs of immune than from normal animals"; while Parker and Franke find "no difference in the bactericidal powers of normal and immune rabbits to intravenous injections of typhoid bacilli." The observations of the latter workers are in accordance with those reported by Bail [3] and our own, although the data presented in their paper, as will be discussed later, are open to serious criticism. On the other hand, we see no justification in the assumption of Stone that the results of Parker and Franke are probably due to the fact "that too many organisms were injected." A careful study and calculation of the figures published shows that Stone injected (with one or two exceptions) one third of a standard blood-agar slant or approximately 460,000 million (?), while Parker and Franke used on the average 39,627,000 (p. 306) to 530-616 million (p. 305) typhoid bacilli. This newly created controversy was investigated and was satisfactorily solved, confirming the findings of Parker and Franke, by a series of additional tests recorded in this paper.

Methods.—The rabbits used in the experiments recorded were all free from coccidiosis; a series in which one animal was found to be infested with these protozoa has been discarded for reasons repeatedly stated in previous papers. With a few exceptions, which are noted, one set of experiments was always conducted on animals of one and the same litter, or a combination of two litters. The latter procedure was necessary only when tests on immunized and normal rabbits were extended over an observation period of 72-96 hours. In order to exclude spontaneous infections with coccidia the litters were raised from does which were known to be healthy, under our own supervision. This likewise excluded to a certain degree the well-known variable resistance to typhoid infections of rabbits of different breeds. Animals of definite age, weight, and of known ancestry were tested with varying amounts of living bacteria. It appears appropriate to emphasize that experimental bacteriologic studies in which the resistance of the animals plays an important rôle should only be conducted on offsprings with a well-known ancestorial history. This fact should govern future experimental work on animals. Some incomplete data at our disposal suggestively indicate that increased resistance to bacterial infections may be inherited; on several occasions it was noted that the offspring of intensively immunized mothers were destroying typhoid bacilli in á shorter time interval than the young of a nonprotected mother. Attention is also called to the family predisposition for urinary infections (Bell and Hartzell[4]).

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[1] Jour. Med. Research, 1919, 39, p. 301.

[2] Jour. Infect. Dis., 1919, 25, p. 284.

[3] Arch. f. Hyg., 1905, 52, p. 272.

The typhoid cultures employed were recently isolated and were either grown on peptic-digest agar or 10% rabbit blood-agar slants. The 18-24 hour old growth was suspended in salt solution strained through cotton, and the number of bacteria per c c of suspension was determined by the dilution plate method. All cultures of one series were made in peptic-digest agar of the same lot. This inexpensive medium surpasses the ordinary veal infusion or meat extract-peptone agar. It is most unwise to express the dosage in slants; uneven growth, as a result of repeated transplanting, etc., produces enormous errors.

In a few experiments a single strain and in others a mixture of 4 to 6 strains was employed. Observations to be reported elsewhere suggested the use of polyhomogenous bacterial antigens. It is selfexplanatory that the immunized rabbits were protected against all the strains included in the test cultures chosen for the infection.

The rabbits were inoculated slowly by various methods specified in the experiments. After varying intervals they were anesthetized with ether, and completely exsanguinated from one or both carotid arteries. The excised organs in toto were placed in Petri dishes and weighed. Pieces of the organs (varying from 0.5 to 6.0 gm.) were then removed, weighed on sterile paper, transferred to sterile mortars and pulped thoroughly with sand. The ground tissues were emulsified in sterile salt solution in the proportion of 1 c c to 100 mg. of tissue. Not infrequently the whole spleen, gallbladder wall, suprarenal, etc., were treated in this manner. The gallbladder wall was carefully dissected from the liver tissue and thoroughly rinsed in salt solution, which was removed by sterile filter paper. The suspensions were prepared without delay after the organs had been excised, and the emulsions were plated in peptic-digest agar in amounts representing from 0.1 to 0.000001 gm. of the organ. Saline was used for the dilution of the emulsion, and by proper shaking it was attempted to obtain a uniform suspension, and a breaking up of the agglutinated bacteria. The plates were counted after 36-47 hours' incubation at 37 C. All figures given in the tables are calculated for 0.1 gm. or 100 mg. of tissue; this number represents not infrequently the average of duplicate or triplicate observations.

Contamination of the plates occurred only in exceptional instances, unless the tissues had been secondarily invaded intra vitam by various types of bacteria. B. bronchisepticus in the lung cultures of rabbits was the only organism which required the use of slide agglutination tests for identification, and it invariably necessitated a repetition of the experiment. Streptococci in the gallbladder or kidney were readily differentiated. All experiments recorded have been performed in duplicate or triplicate, but only one experiment of each series illustrating the result will be given in detail in the tables of this paper.

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[4] Jour. Infect. Dis., 1919, 24, p. 628

THE LOCALIZATION AND SUBSEQUENT DISAPPEARANCE OF B. TYPHOSUS FROM THE ORGANS OF NORMAL RABBITS INOCULATED BY WAY OF THE BLOOD STREAM

The studies of Wyssokowitsch,[5] Bail,[3] Arima,[6] Bull,[7] and Parker and Franke [1] dealing with the course of a typhoid bacillus infection in rabbits have established the fact that the inoculated bacteria accumulate most abundantly in the liver, next in the spleen and bonemarrow. Destruction of the micro-organisms apparently takes place in the blood of the general circulation, through the action of the leukocytes, or the endothelial cells which line the capillaries most numerously in organs where the bloodstream is slower (Wyssokowitsch). In the majority of instances the foregoing tests were made on rabbits inoculated by the intravenous route and with comparatively small numbers of typhoid bacilli. Moreover, the period of observation was rarely extended over 24 hours, and little or no attention was paid to the gallbladder and its content. It has been pointed out in the preceding paper, and by Nichols [8] that large doses are necessary to ensure gallbladder infections, and that as a rule the injection of bacteria into the portal system produces a higher percentage of persistent carriers than the usual intravenous injection. The purpose of the experiments reported is to verify these claims.

Exper. 1. Relation of the Number of Typhoid Bacteria Injected to the Number of Bacilli Found in the Tissues and Bile Ten Minutes After Inoculations.—Three rabbits of a litter 4½ months old were injected under ether anesthesia with varying numbers of a polyhomogenous mixture of 4 recently isolated strains of B. typhosus (K, B1, B and I). The exsanguination of the animals began at the end of 10 minutes and was completed in 5-6 minutes. The organs were immediately excised and plated. The results are presented in table 1.

Study of the table clearly shows that the intravenous injection of only a large dose of typhoid bacilli leads to an elimination of organisms in the gallbladder or the hepatic duct bile, and that the distribution of the inoculated bacilli in all 3 animals is strikingly uniform. Even after complete exsanguination, the liver, the spleen, the bonemarrow, the lungs, the kidneys, and the gallbladder wall, in the order mentioned, retained the inoculated typhoid bacilli. Two observations are of importance: First, the total number of bacteria recovered as estimated from the blood and tissue cultures was far below the actual number injected. This fact, originally observed by Bull, definitely indicates that inside of 20 minutes in vivo, and inside of 30-50 minutes in vitro—time which must be allowed for the necropsy and the preparation of the cultures—30-50% of the inoculated viable typhoid bacilli are sufficiently injured by phagocytosis or otherwise, or perhaps so completely clumped, that our plating method fails to record the true number present. For the present it is impossible to state which one of the various factors is responsible, but it should be kept in mind that the endothelial cells can be phagocytically active even for a few hours after the death of the animal. Second, the percentage of bacteria accumulating in the liver depends somewhat on the total number of typhoid bacilli inoculated. For example, in rabbit 1378, which received a whole slant, the liver retained 12,870 million bacteria, or 34% of the total number injected; while rabbit 1377, which received 1/10 slant, registered only 859 million, or 22%; and rabbit 1380 (1/100 slant) showed 6.8 million, or 18% of the total. On the other hand, the spleen registered only 0.5% of the actual number injected. The liver, as a whole, however, retained

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[5] Ztschr. f. Hyg., 1886, 1, p. 3.

[6] Arch. f. Hyg., 1911, 73, p. 265.

[7] Jour. Exper. Med., 1914, 20, p. 237; 1915, 22, pp. 475 and 487; 1916, 23, p. 419; 24, p. 25.

[8] Jour. Exper. Med., 1916, 24, p. 502.

TABLE 1

DISTRIBUTION OF B. TYPHOSUS IN THE RABBIT FOLLOWING INTRAVENOUS INJECTION OF VARYING AMOUNTS OF ORGANISMS

Litter: Injected and Exsanguinated Under Ether.

Tissue	Injection of 1 Slant = 38,500,000,000 Organisms	Injection of 1/10 Slant = 3,850,000,000 Organisms	Injection of 1/100 Slant = 385,000,000 Organisms
	Rabbit 1378 (W. 1775)	Rabbit 1377 (W. 1775)	Rabbit 1380 (W. 1575)
Left and central liver lobe	22,100,000	1,750,000	130,000
Right and central liver lobe	16,800,000	1,350,000	136,000
Gallbladder wall	130,000	18,800	2,100
Bile	75 (0.8 c c)	14 (2.0 c c)	0 (1.5 c c)
Spleen	7,600,000	4,020,000	142,000
Bonemarrow	2,700,000	530,000	52,000
Mesenteric lymph nodes	27,000	1,900	25
Lungs	750,000	48,000	>2,000 (few bronchi septicus col.)
Kidneys	156,000	9,800	300
Urine	47 per c c	0	0
Carotis blood	22, and 14,000,000 per c c 18,000,000	1,310,000 per c c	62, and 98,000 per c c 80,000 per c c
Heart blood	11,000,000 per c c	....	32,000
Duodenal content	125 B. typhosus colonies	Sterile: negative for B. typhosus	Sterile
Sacculus rotundatus	....	....	Neg. for B. typhosus
Appendix wall	500 B. typhosus colonies	150 B. typhosus colonies	1 B. typhosus colony

more than 50% of the number of bacteria demonstrated in all the other organs, blood, and skeletal muscles, etc., combined.

It should be pointed out that the distribution of the retained bacteria was uneven in the various portions of the liver, which was probably the result of an uneven dispersion of bacilli in the blood-stream. Furthermore, the gallbladder as an appendix of the liver receives an equal share of the bacteria accumulated in the liver. This statement is supported by the following figures estimated from a number of counts obtained in this and similar experiments:

Rabbit 1.—0.33 gm. of gallbladder wall retain 23.000 B. typhousus.
0.33 gm. of liver retain 18.150 B. typhosus.
Rabbit 2.—0.21 gm. of gallbladder wall retain 19.740 B. typhosus.
0.21 gm. of liver retain 31.000 B. typhosus.
Rabbit 3.—0.27 gm. of gallbladder wall retain 50.760 B. typhosus.
0.27 gm. of liver retain 65.000 B. typhosus.

An intravenous injection of typhoid bacilli into the rabbit will therefore always lead to an invasion of the gallbladder wall. If a sufficiently large dose is given, the clumped bacteria in the capillaries of the wall will probably not remain innocuous, but may lead to changes which favor a chronic infection of the bile and the biliary system. This conception will be discussed in the course of the analysis of additional experiments.

In the kidneys only a small number of bacteria accumulated. It is, however, shown that the injection of a very large dose of typhoid bacilli leads to an elimination of these bacteria by the urine. This phase of the experiment will be taken up later.

This and similar experiments also demonstrated that a very large number of typhoid bacilli cannot be removed readily from the blood-stream inside of 20 minutes. Smears prepared from the heart blood of such animals revealed a few loosely accumulated bacilli, while preparations made from rabbits injected with a small number of typhoid bacilli usually showed no bacteria, or a few clumps of aggregated bacilli. It is, therefore, obvious that the usual mechanism of removal of typhoid bacilli introduced into the circulation of rabbits by clumping, and subsequent phagocytosis in the organs, so ably investigated by Bull,[9] is considerably impaired, when overwhelming doses are used. The phenomenon of "agglutination" in platelets is probably closely connected with the mixing or dispersion of the heavy bacterial suspension with the blood, and the irregular gathering of the organisms in the lung capillaries; but this entire mechanism will be considered elsewhere more in detail. The inability of the choked organs to dispose of the retained typhoid bacilli constitutes an important factor in the formation of foci, which continue to maintain the bacteria in the blood stream.

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[9] Jour. Exper. Med., 1915, 22, pp. 475 and 487.

These experiments also prove that a fairly large dose of over 1,000 million organisms is necessary to produce in the rabbit an elimination of bacteria in the bile after an ear vein injection. It is a debatable question whether the appearance of typhoid bacilli in the hepatic or cystic bile of rabbits, subsequent to an intravenous injection, can really serve as an indication that such an animal will become a chronic carrier or develop a cholecystitis. It is, however, certain that the body does not free itself by excreting the living bacteria in the bile and urine. The number of organisms so eliminated is, in proportion to the inoculum, very small; in our case, one five-millionth of the infecting dose of bacilli. Assuming for the present that the elimination of bacteria with the biliary secretion alone is responsible for a persistent gall-bladder infection, it is not surprising to record that Nichols[8] Tanabe and Takeuchi[10] and others noted a higher percentage of carriers in rabbits infected by way of the mesenteric than the ear vein. In their tests more organisms appeared in the bile after portal vein injections than after ear vein injections. We have compared the two methods of infection in several experiments, but have been unable to confirm these claims, as is shown by the results reported in the following:

Exper. 2. The Fate of Typhoid Bacilli Introduced Into the Circulation by Way of the Portal Vein and by Way of an Ear Vein. Comparison Between Intravenous and Subcutaneous Mode of Infection.—Two rabbits (1343 and 1342) of the same litter, 211 days old, received injections, using the ear vein, with 5,000 million typhoid bacilli strain K., and one rabbit (1349) received the same amount subcutaneously. Two rabbits (1344 and 1345), also of the same litter, were laparotomized under ether anesthesia and the bacilli were introduced into the circulation by way of a mesenteric vein. The animals were exsanguinated and their organs plated at the intervals noted in the table.

Study of table 2 shows that the distribution of the injected typhoid bacilli was, with 2 exceptions, the same for the 4 rabbits receiving the infective dose by way of the blood stream. Comparatively few bacteria appeared in the bile 10 minutes after the injection; the rabbit injected in the ear vein showed the largest number. The liver of this animal removed from the blood stream 9% of the inoculated organism, while

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[10] Mitt. a. d. Mediz. Gesellsch. z. Osaka, 1910, 9, refer.; Centralbl. f. Bakteriol. I. Ref., 1911, 50, p. 294.

TABLE 2

Fate of B. Typhosus on Intraportal and Intravenous Inoçulation Compared by the Subcutaneous Method of Injection

Litter Experiment: Intravenous injection of 5,000,000,000 organisms.

Tissue	10′	10′	24 Hours	24 Hours	24 Hours
	Intraportal Injection: 1344	Intravenous Injection: 1343	Intraportal Injection: 1345	Intravenous Injection: 1342	Subcutaneous Injection: 1349
Left and central liver lobe	650,000 (800,000)	810,000 (400,000)	2,400 (3,000)	6,000 (7,400)	0
Right and central liver lobe	550,000 (860,000)	660,000 (400,000)	2,000	6,720 (11,000)	0
Bile	5 (0.4 c c)	60 (0.2 c c)	0 (1 c c)	0 (0.8 c c)	No gallbladder
Gallbladder wall	9,400	1,690	2	128	
Spleen	750,000	2,900,000 (2,310,000)	4,410	1,470	0
Bonemarrow	30,000	26,000	23,000,000	410 (380)	0
Mesenteric lymph nodes	380	120 (72)	8	20	0
Lungs	2,500	6,600	10	136	0
Kidneys	450+	590 (420)	0	7	0
Urine	0 (1 c c enriched 0)	....	0 (cocci)	0 (cocci)	0
Testes	45	....	....	....	....
Carotis blood	46,000 per c c	125,000 per c c	2 per c c	3 per c c	0
Heart blood	8,860 per c c (22,300; 840; 5,000)	18,500 per c c (37,000; 2,900; ....)	1 per c c	0	0
Brilliant Green Eosin Plates:					
Duodenum contents	Sterile	1 colony B. typhosus	Sterile	Sterile	Sterile green plates
Duodenum wall	4 colonies pure	150 B. typhosus pure	7 colonies B. typhosus	Negative for B. typhosus	Sterile green plates
Jejunum contents	Sterile	2 B. typhosus pure	Sterile	Negative for B. typhosus	Negative for B. typhosus
Jejunum wall	4 colonies pure	300 B. typhosus + coli	Sterile	Negative for B. typhosus	Negative for B. typhosus
Ileum contents	B. coli	B. aerogenes	1 B. typhosus +++	Negative for B. typhosus	Negative for B. typhosus
Ileum wall (sacculus rotundatus)	3 colonies B. typhosus ∞ B. aerogenes	3 colonies B. typhosus + B. aerogenes	800 B. typhosus +++	Negative for B. typhosus B. coli	Negative for B. typhosus
Appendix contents	B. coli aerogenes	1 colony B. typhosus	1 B. typhosus	Negative for B. typhosus B. coli	
Appendix wall	3 colonies B. typhosus	B. coli aerogenes	55 B. typhosus +++	Negative for B. typhosus B. aerogenes	

the intraportal injected animal accumulated only 6% of the total dose. At the end of 24 hours the gallbladder bile was sterile in the remaining 3 animals. More bacilli accumulated in the lung and spleen of the animal injected by way of an ear vein than in the one infected through a portal vein. It is generally known that after an intravenous injection bacteria accumulate in the lung capillaries, and that intraportal injections lead to a saturation of the vessels of the liver. Rabbit 1345 was slightly toxic, which explains the high count in the bonemarrow, as has already been pointed out by Parker and Franke.[1] Microscopically, this tissue showed necroses, innumerable nests of free and clumped typhoid bacilli, and a marked absence of regeneration of polymorphonuclear leukocytes. The saline extract of the liver of this animal was atoxic. Of some interest were the bacterial counts obtained from the intestinal wall of rabbit 1345, in comparison with rabbit 1342. Numerous hemorrhages in the sacculus rotundatus and the appendix wall indicated vascular lesions in the intestinal mucosa and submucosa. The cultures proved the cause of these hemorrhages to be nests of typhoid bacilli, and supported the observations of Ribadeau-Dumas and Harvier,[11] and of Chirolanza,[12] that the intestinal content of rabbits can become infected from bacterial emboli and hemorrhages in the intestinal wall. It is obvious that the mesenteric vein inoculation was particularly conducive to the localization of clumped bacilli in the capillaries of the intestines. Under particularly favorable conditions (large infective dose) it is readily possible that the gallbladder wall may become similarly infected. Our particular experiment fails, however, to confirm this conclusion. It is not unlikely that postoperative changes (slight peritonitis, biliary stasis, etc.), which develop as a result of the technic employed to introduce typhoid bacilli by way of the portal system, favor the localization of bacteria in the gallbladder, and they may in part explain the results obtained by Nichols.[8] The intoxication of rabbit 1345, which was the largest animal of the series, points toward the explanation offered. Moreover, in our experience postoperative starvation is conducive to intoxication, and the formation of foci in the bonemarrow and biliary system. On the other hand, our results differ in no respect from those reported by Carmichael [13] and by Else.[14] Both experimenters show that organisms injected into the portal vein do not readily reach the gall-

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[11] Compt. rend. Soc. de biol., 1910, 69, p. 181.

[12] Ztschr. f. Hyg. u. Infektionskrankh., 1909, 62, p. 11.

[13] Jour. Path. & Bacteriol.; 1903, 8, p. 276.

[14] Surg., Gynec. & Obst., 1910, 11, p. 470.

TABLE 3

Fate of B. Typhosus in Normal Rabbits

Rabbits of the same litter, 5 months old; exsanguinated following injection. Injected intravenously: 4,300,000,000 B. typhosus "Kearney."

Tissue	10′	60′	1 Day	2 Days	3 Days	4 Days	5 Days
	Rabbit 1225	Rabbit 1222	Rabbit 1219	Rabbit 1220 C.	Rabbit 1221	Rabbit 1223	Rabbit 1224
Right liver lobe	1,610,000	420,000	700,000	2,320,000‡	180 (139)¶	370	......#
Left liver lobe	1,130,000	550,000	10,000?	5,640,000	700	112 (106, 200)	2,133
Center liver lobe	1,620,000	380,000	50,400	36,000§	130 (180)	111 (105, 200)‖	56,000
Gallbladder wall	300	310	225,000	64,000	792,000	508,000	112,800
Bile	4 per c c	1?	D† = sterile; enriched: 0	19,000,000 per c c (0.4 c c)	10,550,000 per c c	8,000,000 per c c	2,000,000 per c c
Spleen	1,280,000	370,000	6,700	1,200	118	245	385
Left bonemarrow femur	221,000	125,000	1,400	150	13	4 (10)	7
Right bonemarrow femur	190,000	170,000	1,200	330	37	12	3
Mesenteric lymph nodes	250	24	270	67	175?	60	10-21
Lungs	13,000 right	....	300 right lung 290 left lung	70	6	0	0
Left kidney	5,700	52	11	0	0	1	0
Right kidney	....	10	24	4	0	2	0
Femoral muscle	250	....	900?	0	0	0	0
Thymus	....	....	124	70	4	....	....
Carotis blood	1,000,000 per c c	<100*	1,300 per c c	100 per c c	7 per c c	1 per c c	0
Right ventricle	380,000	....	....	....	....	Sterile	Sterile
Duodenum	6 colonies per loop	0	∞ B. typhosus per loop	∞ B. typhosus per loop	∞ B. typhosus	∞ B. typhosus	∞ B. typhosus per loop
Sacculus rotundatus	0	0	0	∞ B. typhosus per loop	∞ B. typhosus	∞ B. typhosus	30 B. typhosus per loop
Stool in rectum	0	0	0	50 B. typhosus per loop	∞ B. typhosus	2 B. typhosus per loopfull	2 B. typhosus per loop
Urine	Sterile	Sterile	Sterile	Sterile	Sterile	Sterile	Sterile

* Dilution too high. † D = direct quantitative plating. ‡ Kidney lobe: 760,000. § Around gallbladder: 630,000.
¶ Kidney lobe: 180. ‖ Liver around gallbladder: 1,850 + 3,600. # Kidney lobe of liver: 92.

bladder. *From an experimental standpoint the mesenteric vein injection offers, therefore, no advantages over the ear vein injection.*

A subcutaneous injection of a large dose of typhoid bacilli fails to cause a general infection. At the end of 24 hours a small number of the introduced organisms can be found at the site of inoculation, while the viscera by ordinary plating procedures are found to be sterile. This experiment confirms similar ones reported by Doerr.[15]

The next important step in the analysis of the fate of typhoid bacilli inoculated into rabbits consisted of culturing the tissues of nonimmunized animals over a period of at least five days. Such experiments demonstrated the ability of certain organ tissues to dispose of the accumulated bacteria and supplied data which indicated that a prolonged infection of the bile was associated with an involvement of the liver and the gallbladder wall. One of these instructive experiments is reported herewith.

Exper. 3. The Fate of Typhoid Bacilli in Nonimmunized Rabbits.—Seven rabbits of one litter, with an average weight of 1630 gm., 129 days old. were inocnlated into an ear vein with 4,300 million typhoid bacilli strain K., grown on peptic-digest agar. The animals were exsanguinated after 10 and 60 minutes, and after 1, 2, 3, 4 and 5 days, and the excised tissues plated. The results are shown in a concrete form in table 3.

It will be noted from table 3 that within 10 and 60 minutes, even 24 hours, the cystic bile contained very few typhoid bacilli, while the gall-bladder wall recorded for rabbit 1219 a very high count. This observation, together with other data to be reported elsewhere, is in our opinion conclusive evidence that a wall infection frequently precedes the invasion of the bile. Erosion of the capillaries of the gallbladder wall occurred in rabbit 1221. This animal showed a blood-tinged cystic bile with leukocytes and fibrin flakes; the wall itself exhibited patches of necroses and mucosal and subserous hemorrhages. In the other rabbits, aside from a high bacterial count, pronounced vascular changes were not evident, but the liver tissue adjacent to the gallbladder registered a large number of viable bacteria. In the experiment under consideration, at least 48 hours elapsed before the cystic bile was heavily infected, although the hepatic duct bile probably contained typhoid bacilli much earlier as innumerable colonies were present on the plates prepared from the duodenum of rabbit 1219. It may be mere coincidence, but the gradual diminution in the number of bacilli per c c of bile in the rabbits sacrificed on the 4th and 5th day is noteworthy, and may be explained by the more active evacuation of the stagnant biliary secretion, stimulated by the digestive activities of the intestinal tract, which was seriously impaired during the intoxication of the animals in the first 3 days following the injection.

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[15] Centralbl. f. Bakteriol., I O., 1905, 39, p. 624.

Aside from the points just mentioned, the results recorded for this and similarly conducted experiments did not as a whole differ from those reported by various writers repeatedly mentioned. The liver retained, 10 minutes after the injection, about 20% of the total number of bacteria introduced into the blood stream, while all the other organs, particularly the kidneys, lymph nodes, muscle, etc., retained very few. The bactericidal action of the serums of rabbits 1225 and 1222 was comparatively low; 1 c c of serum destroyed in 5 hours only 10,000 typhoid bacilli stain K. This may in part explain the high bacterial count of the blood, and the fact that clinically the animals of this series were in comparison with others only slightly intoxicated. This observation is also supported by the low bonemarrow counts. The speed of destruction is influenced by the resistance of the rabbit, and the number of bacteria inoculated, as is shown by the fact that the liver in rabbit 1222, at the end of one hour following the inoculation, still retained 5% of the total number of bacteria, or about one-fourth of the number of typhoid bacilli which originally lodged in this organ.

Aside from the liver, the gallbladder and its contents, the spleen and bonemarrow may retain viable bacteria at a period when the blood is already sterile, and may remain the site of vegetating typhoid bacilli for from 20-30 days. As a rule, the spleen destroys typhoid bacilli more slowly than the liver, a fact already noticed by Werigo.[16] Simultaneously with the disappearance of the germs from the blood, which depends on the bactericidal substances evolved by the tissues and the number of bacilli injected, the kidneys and lungs are found free, while the mesenteric lymph nodes, probably one account of secondary invasion through lacteals of the intestines, remain more or less infected.

These experiments conclusively demonstrate that the typhoid cholecystitis in normal, healthy rabbits is probably not infrequently the result of capillary bacterial emboli in the gallbladder wall, and that a pericholangitis, or a hepatitis, as has been stated by E. A. Graham[17] is a rather constant accompaniment of this process. This phase of our problem will, however, be considered in the next paper of this series.

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[16] Ann. de l'Inst. Pasteur, 1892, 6, p. 478.

[17] Surg., Gynec. & Obst., 1918, 27, p. 521.

A COMPARISON OF THE FATE OF TYPHOID BACILLI IN NORMAL AND IMMUNIZED[18] RABBITS

It has already been stated that Bail, Parker and Franke, and Stone studied the bactericidal powers of normal and immunized rabbits infected by intravenous injections. It has also been pointed out that the results of these workers were obtained by various procedures and that they are not strictly comparable. Bail, for example, worked with rabbits, which were passively immunized by the injection of immune serums. He stated that he encountered considerable difficulty in obtaining uniform results. Parker and Franke prepared their immunized animals with killed and living typhoid bacilli, while Stone inoculated her rabbits with dead bacilli only. Parker and Franke tested their animals with a comparatively small number of typhoid bacilli, while Stone used the profuse growth of blood-agar slants. It naturally suggested itself to investigate whether the various results recorded by these workers were attributable to these differences in technic or to other unknown factors. Our experiments were conducted with these problems in mind, and covered, therefore, three phases: 1. What is the fate of typhoid bacilli intravenously injected into rabbits immunized by dead or living vaccines? 2. Are typhoid bacilli grown on peptic digest agar less readily destroyed by the bactericidal powers of the rabbit's blood and tissues than those grown on rabbit blood agar.[2] 3. Does the dosage of bacteria injected materially influence the end result, and does the immunized animal really destroy typhoid bacilli better and in a shorter time interval than the normal rabbit? The respective experiments to answer these questions were extensive, and our conclusions were based on several hundred animals. For the sake of brevity an example of each type of test will be selected for consideration and discussion.

Exper. 4. The Fate of Typhoid Bacilli in Normal Rabbits and in Those Immunized by Heat-Killed Tricresolized Vaccines Tested by a Moderately Large Number of Bacteria.—Five rabbits of two litters of 11 animals, with an average weight of 2,090 gm., and about 145 days old, were immunized by six intravenous injections of polyhomogenous heat-killed, tricresolized vaccines (13 and 14). Forty days after the last injection these rabbits, together with 6 control animals, of an average weight of 2297 gm., were infected by an intravenous injection of 3,300 million B. typhosus strain K. The rabbits were exsanguinated in pairs after 10 minutes, 5 hours, 1, 2, 4 and 6 days, and the excised organs plated. The counts of viable bacteria are shown in table 4.

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[18] Rabbits are immune against the disease typhoid fever, but they can be immunized against the B. typhosus. In order to emphasize this difference the term immunized instead of immune is chosen.

TABLE 4

The Fate of Typhoid Bacilli in Normal Rabbits and in Those Immunized by Heat-Killed Tricresolized Vaccines Tested by a Moderately Large Dose of Bacteria

Tissue	10′	10′	5 Hours	24 Hours	24 Hours	48 Hours	48 Hours	96 Hours	96 Hours	144 Hours	144 Hours
	Immune Rabbit 1283	Normal Rabbit 1284	Normal Rabbit 1285	Immune Rabbit 1274	Normal Rabbit 1278	Immune Rabbit 1275	Normal Rabbit 1279	Immune Rabbit 1276	Normal Rabbit 1280	Immune Rabbit 1277	Normal Rabbit 1281
Agglutination	1:2000	<1:10	<1:10	1:10,000	<1:10	1:20,000 +++	<1:10	1:10,000- >1:20,000	<1:10- 1:8,000 +++	1:8,000- >1:20,000	<1:10 1:4,000
Liver, left and central	910,000	910,000	4,000-8,000	280	450	210	68-90	132	5,800	10	0
Liver, central and right	1,080,000	830,000	4,500	230	380	270	80	200-260	6,400	8-20	2
Gallbladder wall	4,100	730	9	0	2	0	0	0	82,000	0	0
Bile	11 (0.6 c c)	0 (also enriched)	169 (1.6 c c)	0 (1.8 c c)	0 (0.5 c c)	0 (0.6 c c)	0 (0.9 c c)	0 (1 c c)	103,500,000 p. bile	0 (1.3 c c)	0
Spleen	360,000	230,000	37,000	>3,500	200	201-500-350	104	1,120-1,000	3 (enriched 0)	138-240	0
Bonemarrow pooled	>130,000+	3,100-5,100	>50,000	1,100	50-80	70	6-8	54	1	4	0
Mesenteric lymph nodes	280	60	90	2	52	82	6	105-160	0	1	0
Lungs	12,000	650	200-1,000	70-100	20	0	0	130-200	0	1	0
Kidneys	540	55	17	1	0	0	0	0	910	2,700	0
Urine	0	....	0	260{210 }310	0	......	0	0	++	33 (in 1 c c)	0
Heart blood	0?		100	......	......	0	0	0	0	0	0
Carotis blood	750	100 2,130	400	0	?	1 in 4 plates	R. & L. V. 1	1 in 3 plates	1 in 3 plates	2 in 3 plates	0
Whole blood clotted	230-40-20-72 (5 hours)	10-20-0 (5 hours)	40-20-0 (5 hours)	0	6						

96 Hours	
Normal Rabbit 1280	
Duodenum	∞ B. typhosus
Jejunum	∞ B. typhosus
Colon	∞ B. typhosus
Sacculus rotundatus	B. typhosus
Stool from rectum, direct	Negative
Bile enriched	B. typhosus
Biliary passages	4,040,000
Liver around gallbladder	21,200

144 Hours	
Immune Rabbit 1277	
Duodenum	Negative for B. typhosus
Jejunum	Negative
Ileum	Negative
Sacculus rotundatus	Negative
Cecum	Negative

144 Hours	
Normal Rabbit 1281	
Duodenum	Negative
Jejunum	Negative
Ileum	Negative
Sacculus rotundatus	Negative
Cecum	Negative

Table 4 shows that the ratio of distribution of the typhoid bacilli in the organs of immunized rabbits is practically the same as in the normal animal. In this particular experiment the spleen, bonemarrow and lung of the immunized rabbit sacrificed 10 minutes after the injection, retain more bacteria than the normal. In other experiments this difference is constant only for the lung: This organ may in animals with an acquired immunity seize from 3 to 200 times as many bacteria as that of the normal rabbit. Rather constant is the observation that the splenic tissues of the vaccinated animals retain the typhoid bacilli injected for a longer period, and in somewhat greater number than the normal. These are the only striking differences between the normal and immunized rabbit which have occurred regularly in similarly conducted experiments. The blood serums of the normal rabbits of the two litters exhibited exceedingly high bactericidal properties (see figures on whole blood clotted of rabbit 1284 and rabbit 1285), which in part explains the disappearance of the injected bacteria from the blood stream, irrespective of their low agglutinin content. It is noteworthy that on the sixth day the tissues of normal rabbit 1281 were, with exception of the liver, found sterile, while the immunized animal (rabbit 1277) of the same litter and period of infection, showed typhoid bacilli in most of the organs and even in the blood. The latter finding is probably connected with the persistence of viable organisms in the spleen.

Localization in the gallbladder took place in only one animal (1280). The inflammatory reaction of the gallbladder wall was severe. Indeed, fibrin deposits connected the serosa of the viscus with the stomach wall. An edematous infiltration extended along the cystic duct to the common and to the hepatic biliary passages. Flocculent, slimy débris blocked the common duct and in part explained the high bacterial count of the bile removed from the gallbladder. Judging from these and similar findings, one is again inclined to attribute the cholecystitis to a primary infection of the wall with subsequent invasion of the bile. Attention is also called to the presence of typhoid bacilli in the urine of 3 animals (rabbits 1274, 1280, and 1277). In 2 rabbits this persistence of organisms is associated with a localization of bacteria in the kidneys. Similar observations have indicated that the presence of typhoid bacilli in the urine is frequently characteristic for animals of the same litter, and we therefore suspect an hereditary susceptibility of these organs. Moreover, the typhoid bacilli can multiply in the urines of some rabbits, while in others the findings of these germs is always closely connected with foci in the kidney parenchyma.

The method of immunization, whether accomplished with living or dead organisms, has no influence on the destruction of typhoid bacilli in the blood and tissues of the protected rabbits, as is illustrated by experiment 5, which is again chosen from a number of similar tests.

Exper. 5. The Fate of Typhoid Bacilli in Rabbits Immunized With Living Vaccines and Tested by the Injection of a Moderately Large Dose of Bacteria.— Three members of a litter of 7 rabbits, with an average weight of 1,700 gm., and 132 days old, were immunized according to the method of Bull[19] with a mixture of 4 strains (Bl., B., K. and I.) of living typhoid bacilli. Twenty-eight days after the last injection these rabbits, together with the 4 controls of the same litter, were injected intravenously with 4,100 million of a B. typhosus mixture (strain Bl., B., K., and I.). The distribution and localization of the injected bacteria were studied in one normal rabbit. The remaining 6 animals were sacrificed in pairs after 8, 24, and 72 hours, and the organs plated. The observations are summarized in table 5.

A study of table 5 shows that the serums of the nonimmunized members of this litter destroyed typhoid bacilli rather slowly. The immunization rendered the blood nonbacteriolytic in the test tube, and yet in 2 rabbits killed on the 24th and 72nd hour after the injection, the blood stream harbored fewer organisms than in the normal animals (3 and 1 to 44 and 75). On the other hand, the splenic pulp of the immunized rabbit retained from 2 to 20 times as many bacilli as the normal. The lung tissue of the immunized animal retained on the 8th hour 3 times as many bacteria as its liver, and 60 times as many as the lung of the normal control rabbit. Gallbladder infections resulted in only one normal animal. The number of organisms determined in 1.6 c c of bile was 490; the gallbladder wall registered 31 colonies per 100 mg. of tissue. This particular animal showed numerous infarcts in the liver, and in comparison with other rabbits it was obvious that the presence of a small number of typhoid bacilli was probably the result of biliary elimination from these liver foci. Both the normal and immunized rabbits sacrificed on the 8th hour had bacilli in the bile (10 and 36 per 1 c c). This and similar observations indicate that the mere presence of typhoid bacilli in the gallbladder bile does not lead to a prolonged infection of this viscus.

[19] Jour. Exper. Med., 1916, 23, p. 419.

TABLE 5

THE FATE OF TYPHOID BACILLI IN RABBITS IMMUNIZED WITH LIVING VACCINES AND TESTED BY THE INJECTION OF A MODERATELY LARGE DOSE OF BACTERIA

Tissue	10′	8 Hours	8 Hours	24 Hours	24 Hours	72 Hours	72 Hours
	Normal Rabbit 1327 B	Immune Rabbit 1327	Normal Rabbit 1329	Immune Rabbit 1328	Normal Rabbit 1332	Immune Rabbit 1330	Normal Rabbit 1331
Agglutination	<1:10	1:10,000	1:10	1:8,000	1:10	1:8,000-10,000	1:60-100
Left and central liver	780,000	42,000	60,000	1,360	7,900	62	780 } In-
Right and central liver	750,000	36,000	120,000	1,460	8,100	46	1,200 } farcts
Gallbladder wall	5,500	180	220	0 (cocci)	10	0 (cocci)	31
Bile	36 per c c (1.0 c c)	10 per c c (1.1 c c)	0 (1.2 c c)	0 (0.8 c c)	0 (1 2 c c)	0 (1.4 c c)	490 per c c (1.6 c c)
Spleen	1,610,000	20,000	1,100	4,760	4,100	6,160	2,500
Bonemarrow	364,000	37,000	40,000	2,560	8,000	20	190
Mesenteric lymph nodes	1,700	152	80	152	95	160	300
Lungs	10,000	107,000	1,680	750	300	2 (B. bronchi-septicus)	1 (B. bronchi-septicus)
Kidneys	13,200	2,800-3,200	170	19	10	<10	2
Urine	0 (3)	0	0 (3)	0 (3)	0 (1)	0 (3)	3,450,000 per c c
Thymus	1,050	....	....	....	....	....	....
Carotis blood	I 2,325,000 per c c II 1,620,000 per c c	I 265 per c c II 225 per c c	I 105 per c c II 85 per c c	I 11 per c c II 4 per c c	I 44 per c c II 39 per c c	0, 0, 1 per c c	I 74 per c c II 56 per c c
Heart blood	....	120 per c c	210 per c c	3 per c c	34 per c c	0 per c c	75 per c c
Duodenum	1 colony of B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus
Jejunum	....	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus
Ileum	....	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus
Cecum	....	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus
Appendix wall	2 colonies	61 B. typhosus colonies	Negative for B. typhosus	Negative for B. typhosus	1 B. typhosus	Negative for B. typhosus	Negative for B. typhosus
Appendix contents	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	2 B. typhosus ulcer in colon ∞ B. typhosus	Negative for B. typhosus	Negative for B. typhosus

A bacilluria of 3,400,000 typhoid organisms per c c with a few bacteria in the kidneys (2 per 100 mg.) was noted in a normal rabbit killed on the third day. The liver of the normal rabbit killed on the 8th hour contained toxins demonstrable by the method of Parker, while the saline extract of the same organ of the immunized animal was innocuous. On several occasions we tested the fate and destruction of typhoid bacilli in rabbits which were gallbladder-typhoid carriers. The removal of the organisms from the blood by the tissues, and their bactericidal action proceeded in these animals in an identical manner, as observed in control rabbits studied at the same time under identical conditions.

The experiments of Parker and Franke were conducted on rabbits immunized with living typhoid bacilli, while Stone tested animals vaccinated with heat-killed preparations. The only additional differences in the experiments of these workers was the use by Stone of typhoid bacilli grown on rabbit-blood agar slants. Typhoid bacilli acquired on this medium, particularly when transplanted at regular intervals of from 24-48 hours, certain properties which apparently are conducive of gallbladder infections. Physical, as well as biologic factors, which we have not been able to study with the desired thoroughness, are probably responsible for these properties. The growth energy of B. typhosus is enhanced on blood mediums and with it also the pathogenic powers, particularly the parasitic function. This applies in a lesser degree to the toxicogenic properties. In many respects this behavior of typhoid bacilli frequently transferred on blood mediums is analogous to the changes in virulence observed by Wadsworth and Kirkbride [20] for the pneumococccus. From several experimental series in each of which 2 normal rabbits were infected with typhoid bacilli grown on rabbit-blood agar (35 generations) and 2 others with the same number of bacteria reared on veal-infusion agar (same number of generations), it must be concluded that no difference in the distribution and localization or destruction of the germs is demonstrable. Toxic symptoms and a high bonemarrow count at the end of the 24th hour were, however, noted in several animals injected with the veal-agar growth. These observations suggest a number of tests on immunized rabbits and one experiment of a series of 3 is chosen for discussion.

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[20] Ibid., 1918, 28, p. 791.

TABLE 6

THE FATE OF B. TYPHOSUS GROWN ON RABBIT-BLOOD AGAR IN THE IMMUNIZED AND NORMAL RABBIT

Tissues	10′	24 Hours	24 Hours	96 Hours	96 Hours	120 Hours	120 Hours
	Normal Rabbit 1595 o+	Immune Rabbit 1646 o	Normal Rabbit 1522 o+	Immune Rabbit 1591	Normal Rabbit 1686*	Immune Rabbit 1589	Normal Rabbit 1593
Agglutination titer	1:10++	1:40,000	1:10	1:60,000+++	1:40+++	1:40,000+++	1:800+++
Left and central liver	1,200,000	840	300	30,000	800,000	60,000	1,600
Right and central liver	2,000,000	760	1,210	1,100	840,000	50,000	630
Bile	1,334 (0.5 c c)	0 (enriched +) 0.05 c c	0 (1.2 c c)	1,420,000 (0.9 c c)	960,000,000 per 0.8 c c	58,000,000 per 0.7 c c	50,000 per 0.8 c c
Gallbladder	7,200	10	0 difco streptococci	19,000	5,500,000	230,000	44,000
Spleen	900,000	1,500	400	24	17,000	1,600	410
Bonemarrow	80,000	5,500	5,000	36	6,000,000	24	58
Mesenteric lymph nodes	15,000	17	18	22	....	3	21
Lungs	22,000	7,000	3,000	250	1,800	150	7
			3	180	267,000	0	12,000
Kidneys	62,000	9				(enriched ++)	
Urine	3, 2 per c c	0, 0	169 per c c	0: 0	10	0	66,000 per c c
Carotis blood	4,800,000; 6,000,000	35; 20; 14; 20; 23; 14	76; 65; 140; 55; 90	0; 0; 0	....	0; 0; 0; 0; 0; 0; 0	0; 0; 1; 0; 0; 1; 0
Heart blood, left vent.	4,900,000	12: 10	66; 40	0; 0; 0	8 partially coagulated blood	0; 0; 0	3; 0; 0
right vent.	3,000,000	20; 14	40; 130; 90	0; 0; 0	....	0; 0; 0	0; 0; 1
Duodenum	6 colonies pure	Negative for B. typhosus	Negative for B. typhosus	∞ B. typhosus colonies	∞ B. typhosus colonies	5 B. typhosus colonies	∞ B. typhosus
Sacculus rotundatus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	1 B. typhosus colony	∞ B. typhosus colonies	8 B. typhosus colonies	Negative for B. typhosus

* Died shortly before cultures were made.

Exper. 6. The Fate of B. Typhosus Grown on Rabbit-Blood Agar in the Immunized and Normal Rabbit.—Three rabbits of a litter of 7 animals, with an average weight of 2,500 gm., and about 170 days old, were immunized with a mixture of 7 strains of heat-killed and living typhoid bacilli in a similar manner as that described in exper. 5. Fifteen days after the last injection these animals, in addition to the remaining 4 members of the litter, were infected by an intravenous injection of 4,300 million of a polyhomogenous saline mixture of 7 typhoid strains grown on 10% rabbit-blood agar. The cultural findings are shown in a concrete form in table 6.

Attention is called to the fact that the immunized and normal rabbits exhibited pronounced localization of the typhoid bacillus in the gallbladder and bile 96 and 120 hours after the introduction of the infective dose, while the animals killed on the 24th hour gave sterile cultures from this viscus. These observations indicate that blood-agar cultures are suited for the production of biliary infections, but they also confirm the conclusion reached from exper. 3, that the bile or the gallbladder does not necessarily become infected in the first 24 hours. It may also be assumed that elimination of typhoid bacilli by the hepatic duct bile in the first 10 minutes does not necessarily lead to a prolonged infection of this viscus. The evidence in this and similar experiments is decidedly in favor of the conception that, in the rabbit intravenously inoculated, not infrequently an infection of the gallbladder wall, or extensive liver lesions, initiate the invasion of the bile. It should, however, be emphasized that in this respect no difference exists between the immunized and normal rabbits. Anatomically, hemorrhages and even slight erosions of the gallbladder mucosa are distinctly visible; every rabbit shows, also, extensive liver necroses. In rabbit 1589 an obstruction of the common duct produced a uniform dilatation of the bile ducts which explained the high bacterial count of the liver tissue, while in rabbit 1593 the washed gallbladder wall registered practically the same number of typhoid bacilli as 0.8 c c of bile.

The bacterial count of the bonemarrow of rabbit 1686 indicates the existence of a focus, which led, directly or indirectly, to the intoxication and to the death of the animal. The high bacterial counts of the tissues must be partially attributed to a multiplication of the typhoid bacilli in the blood stream during a rather prolonged agonal stage of the disease, and partially to the fact that toward the termination of the infection the organs, like the liver, the spleen and the lungs, primarily engaged in the destruction of the bacteria, are overwhelmed with organisms brought to them from the bonemarrow foci. It is known from the statements already made that 3-4 days usually elapse before the blood of the normal, and frequently also the immunized, rabbits is found sterile. In our experience with a considerably larger number of animals than reported by Stone, we have been unable to confirm her conclusions that the blood of typhoid immunized rabbits sterilizes itself in 18 hours. Heart-blood cultures, of course, should be chosen for this comparison. In rabbits showing extensive liver or splenic foci the carotid blood toward the end of the exsanguination may record a few typhoid bacilli. Furthermore, sterility of the blood is in no way dependent on its content of immune agglutinins or other antibodies. Rabbits immunized with sensitized bacteria, as a rule, lack agglutinins, although they are similarly resistant to an infection as are rabbits protected with living bacteria.

Most of the rabbits in this experiment possessed at necropsy a distinct splenic tumor, and this tissue in the immunized rabbits retained slightly more bacilli per 100 mg. of organ than the normal ones. For reasons already given, the pair of rabbits killed on the 96th hour cannot be included in these deductions.

Localization in the kidneys, early elimination, and even persistence of the typhoid bacilli in the urine, were characteristic for this litter. The latter condition might be due in part to a change in the reaction and composition of the urine. Complete disappearance of the urinary carbonates and a distinct reduction in the alkali blood reserve occurred in the rabbits with urinary infections.

Briefly summarized, it can be stated that the intravenous injection of normal and immunized rabbits with typhoid bacilli grown on rabbit-blood agar failed to demonstrate differences in the destruction and the distribution of the bacteria. It was, however, noted that localization in the gallbladder and bile 96-120 hours after the injection occurred more readily with moderately heavy suspensions prepared from rabbit-blood agar slants than with those cultivated on peptic-digest agar.

In accordance with the plan of our investigation it was necessary to verify the interpretations of Stone: Can an immunized rabbit dispose more rapidly of a small number of typhoid bacilli than a nonimmunized one: and are the results published by Parker and Franke really explained by the fact that these workers employed as an infective dose less than 1 billion bacteria? In three series of experiments these questions were investigated. The number of organisms injected varied from 215-562 million bacteria. Litters of different ages were chosen, and it was conclusively demonstrated that no differences existed in the normal or immunized rabbits, either in the destruction or in the localization of such numbers of typhoid bacilli. Two hundred and fifteen million were disposed of in a shorter time interval than 562 million, but the immunized animal differed in this respect in no way from the normal. Moreover, the variable resistance of different litters, sometimes influenced by B. bronchisepticus infections, was more readily demonstrable following the injection of small doses of typhoid bacilli, but in no case were these variations greater in immunized than in normal animals. The same deduction applies to the apparent differences depending on the toxicity or virulence of the individual typhoid strains. The correctness of these conclusions is illustrated by the description of one experiment.

Exper. 7. The Fate of a Small Dose of B. Typhosus in Robbits Immunized With Dead and Living Organisms.—Three rabbits of one litter and 3 of another, all 197 days old, and of an average weight of 1,991 gm., were immnnized either by Bull's method with a polyhomogenons mixture of typhoid bacilli, or by 6 injections of a heat-killed, tricresolized vaccine. Seventeen days after the last injection, these rabbits, together with 3 controls (members of the litter immunized with dead bacteria) were injected with 350 million typhoid bacilli (7 strains). The cultivating of the organs was conducted as usual. Table 7 presents the results in concrete form.

The figures presented in table 7 seem to indicate that the number of typhoid bacilli inoculated has no appreciable influence on the mechanism responsible for their progressive destruction in the rabbit body. It may be mere coincidence, but in this and similar experiments, it was noted that frequently the normal rabbit sterilized itself in a shorter time interval than the immunized animal. The bacterial counts of the spleen and bonemarrow should be chosen for such comparison. In the experiments under consideration, Rabbit 1644, possessing a gallbladder infection probably contracted during the immunization with living organisms, disposed rather slowly of the inoculated bacteria. Furthermore, experiments suggest that the blood stream will remain free from viable typhoid bacilli when the spleen has completely disposed of the accumulated microbes. Infarction of the spleen with marked enlargement and a poor exsanguination are some of the factors responsible for the high bacterial count in rabbit 1588.

Certain observations pertaining to the localization of the inoculated bacilli deserve some brief consideration. The liver retains in the first 10 minutes after inoculation from 10 to 20% of the injected organisms. The figures dealing with the lung tissues are also of interest. The lungs of rabbit 1647, immunized with living bacteria, removed from the blood stream about 100 times as many bacilli as the normal, and 10

TABLE 7

The Fate of a Small Dose of B. Typhosus in Rabbits Immunized With Dead and Living Organisms

Tissues	10′			24 Hours			72 Hours		
	Immune Rabbit 1580	Immune Rabbit 1647	Normal Rabbit 1583	Immune Rabbit 1581	Immune Rabbit 1644	Normal Rabbit 1584	Immune Rabbit 1585	Immune Rabbit 1645	Normal Rabbit 1588
Mode of immunization	Heat killed and tri-cresolized (7 injections)	Heat killed and living (7 injections)	....	Heat killed and tri-cresolized (7 injections)	Heat killed and living (7 injections)	....	Heat killed and tri-cresolized (7 injections)	Heat killed and living (7 injections)	....
Agglutination titer....	1:200-400+++	1:6,000+++; 1:20,000+++	<1:10	1:6,000+++	1:8,000-1:10,000+++	<1:10	1:80+++	1:4,000+++ 1:8,000++	<1:10
Left and central liver..	58,000	95,000	30,000	250	4,500	10	5	3	12
Right and central liver	130,000	120,000	6,000	630	19,000	<10	2	1	16
Bile....................	....	0 (0.5 c c)	0 (0.6 c c)	0 (0.1 c c)	12,000,000 (0.9 c c)	0 (0.2 c c)	0 (0.2 c c)	0 (0.8 c c)	0 (0.4 c c)
Gallbladder wall.......	Absent	700	135	1	39,000	0	0	0	0
Spleen..................	40,000	13,000	17,200	180	18	<10	70	19	2,650
Bonemarrow...........	6,800	7,000	2,000	400	6	1	0	0	3
Mesent. lymph nodes..	1	6	0	0 (cocci)	25	2	1	0	0 (coli)
Lungs..................	230	2,400	20	10	1	10	0	0	1
Kidneys................	0 (20,000 streptococci)	42	11	1	1,130	0	0	0	0
Urine...................	0, 0, 0	0, 0, 0	0, 0, 0	0, 0	0, 0, 0	0, 0, 0	0, 0, 0	0, 0	0, 0
Carotis blood..........	80, 80	400	2,800, 1,300	0, 0, 1, 0, 1	0, 0, 0, 0, 0	0, 0, 0, 0	0 (E:O)	0 (E:O)	0, 0, 1, 0, 0, 0, 0
Heart blood...........	120, 100	900, 100	700, 600	0, 0, 0, 0	0, 0, 0 (E:O)	0, 0, 0, 0 (E:O)	0 (E:O)	0 (E:O)	0, 1, 0, 0, 0
Duodenum.............	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	∞ B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus
Sacculus rotundatus...	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus	Negative for B. typhosus

times as many as rabbit 1580, which was only treated with heat-killed vaccines. As a rule, this behavior of the lung tissue is constant in healthy, immunized rabbits, but may be seriously disturbed by a latent B. bronchisepticus infection, so frequently encountered in animals subjected to bacterial vaccines or their split products. Extensive immunization, however, is conducive to a deposition of bacteria in the pulmonary capillaries. Just why they are removed by the lung of the immunized or sensitized animal we have not as yet determined conclusively, but the recent observations of Coca [21] on sensitized rabbits suggest that an occlusion of the pulmonary vessels is perhaps the most important factor. Most of the investigators dealing with the distribution of bacteria in the normal lung of animals following an intravenous injection conclude that the organisms accumulate in these tissues as a result of mechanical influences, notably the difficulty of dispersing the bacteria in the blood, the small size of the capillaries and the secondary accumulation of leukocytes. According to our observations, neither the dispersion nor the accumulation of leukocytes is notably different in the immunized rabbits, but the lumen of the capillaries is narrowed and assists in the retention of the injected organisms.

In comparing our data with those published by Parker and Franke, we note that as a whole the results of the experiments on immunized and normal rabbits are identical. However, certain figures in their article appear to be incorrect. For example, on page 306 they state that a normal rabbit (1), weighing 1,900 gm., was injected with 39,627,000 typhoid bacilli; the liver retained 15 minutes after the inoculation 270,000 bacteria per 100 mg. of tissue; calculated for the liver weight [22] of this animal, this organ alone would have retained 153,000,000 bacteria. A similar discrepancy exists between the inoculum and the liver count for an immune rabbit 2. The inoculation was stated to be 39 million typhoid bacilli, while based on calculations, the liver removed in 10 minutes 287 million. Throughout this publication the percentage of bacteria retained by the liver tissue varies between 30-60%. These figures are, according to the data furnished by Arima [23] and our own, unquestionably too high, and it appears not unlikely that the counts dealing with the test dose are incorrect. The more recent work of Stone is similarly invalidated by incorrect determinations of the inoculated bacteria. According to her estimates, 460,000 million, or 460 billion were inoculated, and yet at the end of 5 hours the blood stream of one rabbit contained only 30 bacteria per c c. In view of these discrepancies, it is justifiable to assume that in all probability not more than 4,600 million bacteria were actually inoculated. Moreover, in the light of the data of Bail [3] and our own, it appears utterly impossible that the blood and tissues of an immunized rabbit can destroy in 18-24 hours 460 billion typhoid bacilli. The findings of Stone are certainly "paradoxical," to use her own expression, but one is unable to explain the sources of error on account of the sketchy character of the experimental data. It is not unlikely that the method of incomplete tissue cultivation or the preparation of the tissue suspensions is at fault.

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[21] Jour. Immunol., 1919, 4, p. 219.

[22] We have estimated the liver weight of 200 normal rabbits as 1/30 of the body weight.

[23] Arch. f. Hyg., 1911, 73, p. 265.

The results of our experiments with typhoid bacilli intravenously inoculated into normal and immunized rabbits can be briefly summarized:

1. A small number of bacteria is rapidly removed from the blood stream; about 20 to 30% of the inoculum can be found in the liver, a smaller percentage in the spleen, bonemarrow and lungs, and comparatively small numbers in the lymph nodes, kidneys, muscle, etc. The gallbladder bile contains a small number of bacteria, while the gallbladder wall receives, according to its size, a proportional share of the total number of bacteria deposited in the liver. The intraportal injection has no advantage over the intravenous method.

2. Large numbers of typhoid bacilli are less speedily removed from the blood stream, but the distribution in the organs remains the same in the first 4 to 8 hours. The bacteria taken up by the tissues are rapidly reduced in number, particularly in the liver, spleen and lungs. The bonemarrow is less active and not infrequently the typhoid bacilli multiply in these tissues and form foci, which reinfect the blood stream. The gallbladder can become infected either as a result of an immediate extensive elimination of the introduced bacteria, or the continuous discharge of bacilli from liver foci in the hepatic bile, or in consequence of an embolic infarction of the gallbladder capillaries, causing a diphtheric cholecystitis. In the latter instance the cystic bile may not become invaded with typhoid bacilli for 24-48 hours after the intravenous injection.

3. The distribution and destruction of small or large doses of typhoid bacilli is practically the same in the normal as in the immunized rabbit, whether protected by the injection of dead or living organisms.

The only noteworthy difference between the normal and immunized rabbit is found in the lung and spleen; namely, the lung of the immunized rabbit takes up a somewhat greater number of bacteria than that of the normal; and the spleen of the immune sterilizes itself more slowly than that of the normal.

4. Localization in the gallbladder and bile 96-120 hours after the infection, occurs more readily with moderately heavy suspensions prepared from rabbit-blood agar than with those obtained from peptic-digest agar slants.

5. Rabbits which succumb to the typhoid intoxication regularly harbor enormous numbers of typhoid bacilli in the bonemarrow of the long bones; these foci are probably the seedbeds responsible for the continuous invasion of the blood stream and the subsequent overwhelming infection of the liver and spleen.

THE LOCALIZATION AND SUBSEQUENT DESTRUCTION OF PARATYPHOID A BACILLI IN THE TISSUES OF NORMAL AND IMMUNIZED RABBITS INFECTED BY THE INTRAVENOUS ROUTE

It has been stated in the introduction that the paratyphoid A bacillus behaves like the typhoid bacillus in the tissues of rabbits. This conclusion is based on 3 series of experiments—2 conducted with a large number of bacilli (4,500 million and 3,400 million, respectively); and one with a moderately large inoculum (590 million). One experiment is chosen for discussion.

Exper. 8. Five rabbits of 2 litters, about 210 days old, and an average weight of 2,427 gm., were immunized by Bull's method (dead and living bacteria) with a mixture of 3 paratyphoid A strains, isolated from carriers and obtained through the courtesy of Dr. Charles Krumwiede from the New York City Department of Health. Fifteen days after the last injection these rabbits, with an equal number of controls, members of the same litters, were injected intravenously with 3,400 million of a mixture of the paratyphoid A bacilli used for the immunization. After the lapse of 10 minutes, 24, 48, 96 and 144 hours the rabbits were exsanguinated, their organs excised and cultures made by the usual technic.

Two normal and one immunized rabbits showed extensive biliary infections, with correspondingly high counts in the liver tissue. The counts in the splenic tissue and bonemarrow of the normal, as well as the immunized, rabbits were low and practically identical; for example, the spleen cultures showed on the 48th hour for the normal rabbit 79, and the immunized, 70 colonies per 100 mg. of tissue; on the 96th hour for the normal, 106, and 200 for the immunized.

The lung tissue of the immunized rabbit retained 200 times the number of bacteria deposited in the same tissue of the normal. The blood stream of the animals was not sterile before the 96th hour; at the end of 24 hours the blood of the normal animal registered fewer bacteria than the immunized rabbit. Five animals, or 50%, exhibited either renal or urinary bladder infections; the urine of one immune rabbit contained 3,300 paratyphoid bacilli per c c.

The results obtained in these experiments differed in no way from those reported for the typhoid bacillus. The well-known native typhoid immunity, which is apparently responsible for these results in rabbits, is likewise potent for the paratyphoid A. bacillus. The conclusions which have been drawn from the experiments with the typhoid bacillus can be applied unreservedly to the studies conducted with the paratyphoid A bacillus.

THE LOCALIZATION AND SUBSEQUENT DESTRUCTION OF PARATYPHOID B BACILLI (RABBIT AND HUMAN ORIGIN) IN THE TISSUES OF NORMAL AND IMMUNIZED RABBITS INFECTED BY THE INTRAVENOUS ROUTE

It is well known that rabbits may be highly susceptible to paratyphoid B bacilli. One of us (K.F.M.[24]) discussed this subject recently in connection with the description of a spontaneous paratyphoid B epidemic in laboratory rabbits. The isolation of a rabbit-pathogenic organism suggested some experiments along the lines already mentioned for the typhoid and paratyphoid A bacilli. In the course of these tests it was noted that the bactericidal powers of the immunized rabbits excelled those of normal rabbits infected with moderately large doses of virulent animal paratyphoid bacilli. In general the same principle was found to hold true for human paratyphoid B bacilli or B. schottmülleri. One experiment is chosen to discuss this important phenomenon.

Exper. 9. Ten rabbits of 2 litters over 1 year old, of an average weight of 3,080 gm., were used. Five animals were immunized by 3 subcutaneous injections of 0.01, 0.05 and 0.1 c c of a 24-hour old living broth culture of B. aertryckei (strain 1371).[24] Twenty-eight days after the last injection, the serum of these rabbits agglutinated the immunizing strain in dilutions of 1:1000 to 1:10,000, while the nonprotected animals of the same species either failed to clump the antigen, or did so in dilutions not exceeding 1:20. The entire series of rabbits was infected by an intravenous injection of 700 million bacteria of the identical strain, 1371. Exsanguination, excision of the organs and plating were conducted 10 minutes, 4, 24, and 72 hours after the introduction of the infective dose. The remaining normal animal was moribund on the 120th hour and was killed; while cultures were made from the immunized rabbit on the 144th hour. The cultural findings are presented in concrete form in table 8.

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[24] Litch and Meyer, Jour. Infect. Dis., 1920, 28, p. 27.

It is clearly demonstrated in table 8 that the immunized rabbits possess the power to destroy paratyphoid B. bacilli more readily than the nonprotected animals of the same species. These differences are clearly evidenced by the clinical symptoms; the immunized rabbits appear lively and lose little weight, while the normal ones, after an incubation time of at least 24 hours, refuse to eat, exhibit general prostration, labored breathing, severe diarrhea, and loss of from 300 to 500 gm. of body weight. In contrast to typhoid animals in which the evidences of intoxication occur early, these symptoms never develop before the 24th hour. Aside from this striking fundamental difference, there are several other features which distinguish a paratyphoid B infection from the typhoid infection in the rabbit, hence a detailed analysis of the data presented in table 8 is justified.

The ratio of distribution of paratyphoid bacilli in the organs is practically the same as that noted for the typhoid bacillus; again the lung tissues of the immunized animals retain slightly more (2-7 times) organisms than the normal. The predominance of bacteria in the normal spleen is rather constant; this organ not only retains a large number of bacteria, but is frequently unable to dispose of the invaders. In case of recovery from the infection, 15 to 25 days elapse until sterility is established. On the other hand, the spleen of the immunized rabbits becomes free from bacteria in from 6-8 days if less than one billion micro-organisms has been inoculated. Occasional infarction will prolong this period for 10 or more days. These observations furthermore suggest that the persistence of a large number of paratyphoid bacilli in the spleen is in all probability responsible for the organisms in the blood stream. Sterility of the blood in rabbit 1415 was accompanied also by sterility of the spleen and bonemarrow, and was not due to the agglutinin content of the serum. In fact, the importance of the spleen was demonstrated even more distinctly in several additional experiments not reported in this paper. Invariably the number of bacteria found in this organ in normal rabbits shortly before death exceeded that recorded for the liver or bonemarrow. For example, in a moribund rabbit, 1477,

TABLE 8

The Localization and Subsequent Destruction of Paratyphoid B. Bacilli (Rabbit Origin) in the Tissues of Normal and Immunized Rabbits Infected by the Intravenous Route

Tissues	Immune Rabbit 1352 Agglutination 1:8,000		Normal Rabbit 1443 Agglutination 1:60		Immune Rabbit 1353	Normal Rabbit 1445	Immune Rabbit 1416	Normal Rabbit 1447	Immune Rabbit 1444	Normal Rabbit 1414	Immune Rabbit 1415	Normal Rabbit 1446
	10′	4 Hours' Incubation	10′	4 Hours' Incubation	4 Hours in Vivo	4 Hours in Vivo	24 Hours	24 Hours	72 Hours	72 Hours	144 Hours	120 Hours
					1:1,000 3+- 1:4,000 2+		1:8,000 3+		1:6,000 3+		1:8,000 1:10,000	
Left and central liver	111,000	3,400,000	148,000	14,000,000	11,700	118,000	2,720	45,000	4,000 2,850	25,500	230	27,000
Right and central liver	102,000	4,000,000	170,000		11,400	95,000	3,400	41,000	1,500	28,900	570	134,000
Gallbladder wall	156	....	288	....	38	20	13	80	0	10	300,000	18,500,000
Bile	0 (1.5 c c)	....	257 (0.4 c c)	.... .	44,200	0 (0.45 c c)	0 (0.6 c c)	3,800 (1.1 c c)	0 (0.7 c c)	1 (1.13 c c)	270,000,000 (0.5 c c pus)	18,900,000 per c c
Spleen	6,700	6,200,000	186,000	27,500,000	2,350	23,000	135	219,000	5,400	130,000	1	165,000
Bonemarrow	14,100	1,110,000	22,000	3,600,000	8,000	43,000	450	52,000,000	350	21,300	0	100,000
Mesenteric lymph nodes	42	2,180	38	150,000	12	30,000	62	190	5,600	42,300	36	960
Lungs	12,500	24,000	5,000	200,000	342	312	130 (76)	16,000	152	21,800	0 (sterile)	25,000
Kidneys	230	43,000	1,290	400,000	40	57	4	132,000	3	244,000	430	80 000
Urine	0; 0 per c c	....	0; 0; 0 per c c	....	0; 0; 0 per c c	....	....	....	....	....	1,100 per c c	10,000 per c c
Carotis blood	800; 2,800 per c c	....	5,000; 11,000; 9,500 per c c	....	6; 2; 4 per c c	40; 50; 56; 63 per c c	11; 15; 8 per c c	3,500; 4,160; 3,400 per c c	3 per c c	97, 142 per c c	0 per c c	....
Clotted blood	220	136	2,300	600,000	....	....	....	....	....	....	....	....
Heart blood	....	....	....	....	....	....	13 per c c	4,090 per c c	6, 70 per c c	690, 480, 1,200 per c c	0 per c c	600; 2,100; 680 per c c
Contents of:												
Duodenum	Sterile	....	1 B. paratyphoid colony	....	Sterile on green plate	Sterile	Neg. for B. paratyphoid B	Neg. for B. paratyphoid B	Neg. for B. paratyphoid B	∞ B. paratyphoid B	∞ B. paratyphoid B	∞ B. paratyphoid B
Jejunum	....	....	....	....	Sterile on green plate	Sterile	Neg. for B. paratyphoid B	1 colony B. paratyphoid B	Neg. for B. paratyphoid B	∞ B. paratyphoid B	6 B. paratyphoid B	....
Ileum	....	....	....	....	....	....	Neg. for B. paratyphoid B	....	Neg. for B. paratyphoid B	....	....	..
Sacculus rotundatus	....	....	....	....	1,000 B. paratyphoid B	....	25 B. paratyphoid B	10 B. paratyphoid B	Neg. for B. paratyphoid B	∞ B. paratyphoid B	∞ B. paratyphoid B	....
Appendix	....	....	....	....	33 B. paratyphoid B	....	37 B. paratyphoid B	200 B. paratyphoid B	Neg. for B. paratyphoid B	∞ B. paratyphoid B	250 B. paratyphoid B	....

exsanguinated on the 40th hour, the spleen contained 88 million paratyphoid bacilli per 100 mg., the bonemarrow 22 million, the liver 11 million, and the blood 75,000 bacteria. The activities of the spleen are further indicated by the anatomic response of the organ; a spleen tumor, together with an increased weight, is rather constant. The average splenic weight for the immunized animal was 2.61 gm., and for the normal, 2.15 gm., in certain individual cases a weight of 4.25 and 3.36 gm., respectively, was recorded. Compared with the normal weight and estimated per 1,000 gm. of bodyweight, it was calculated that the increase in weight is more marked in the immunized than in normal rabbits, which in turn suggests an increase in cellular elements. It has been shown by F. A. Evans[25] that the acute splenic tumor of rabbits infected with paratyphoid-like bacteria (B. enteritidis) is characterized by hyperplasia of the phagocytic reticulo-endothelial macrophages, and by a decrease in the number of the other cells of the pulp. The latter condition is probably the result of a toxic inhibition of the leukopoietic function and perhaps a stimulation of the functional reticulo-endothelial cells. The lymphatic nodes also exhibit, in certain rabbits, remarkable affinity for the paratyphoid bacilli, the significance of which fact is not quite clear, but deserves further elucidation. In the first 24 hours the differences between immunized and normal rabbits are fairly uniform; the blood, liver and spleen of the latter contain 10 times as many living organisms as the former. For the lung tissue this contrast is not so clearly demonstrated on account of the initial high count. After 24 hours the actual number of bacteria demonstrable in the organs of the individual rabbits may fluctuate considerably and less uniform counts are obtainable. The focal necroses and embolic processes (gallbladder), which develop quite frequently in immunized animals, and the variations in the individual resistance even in litters, are conducive to these differences. Focal necroses in the bonemarrow of rabbit 1447 and bacterial emboli indicated by hemorrhages in the kidney cortex of the same rabbit, as well as in rabbit 1444 and rabbit 1446, are probably responsible for the high bacterial counts recorded .

Two normal and 2 immunized rabbits developed biliary infections. The finding of paratyphoid bacilli in the bile of 2 animals in the first 24 hours is probably the result of an elimination of these organisms by way of the biliary passages, and not the sequel of a gallbladder wall infection, as noted for rabbit 1415 and rabbit 1446. This phase of the

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[25] Bull. Johns Hopkins Hosp., 1916, 27, p. 356

problem has already been discussed by Litch and Meyer in a recent paper on rabbit typhoid (100). These observations likewise confirm the statement that rabbits intravenously inoculated may fail to register bacteria in the bile 24-72 hours after an intravenous injection, and yet may develop a chronic cholecystitis as a result of bacterial embolism of the gallbladder wall. Every nonimmunized rabbit which succumbed to the intravenous infection on the 4th-11th day exhibited a diphtheric, necrotized cholecystitis, although repeated observations in supplementary tests failed to indicate that paratyphoid bacilli are discharged through the bile stream in the first 30 minutes following an intravenous injection of a number of bacteria large enough to insure a general infection and death in from 3 to 5 days. After a lapse of 24 hours bacteria are undoubtedly eliminated by the bile, as is suggested by the positive cultures obtained from the duodenal contents of rabbit 1444, while the gallbladder is sterile, or nearly so, and the wall of this viscus contains a few bacteria corresponding entirely to the degree of the general blood infection. In this connection another difference between the typhoid and paratyphoid infection of the rabbit is noted. The contents of the ileum, sacculus rotundatus and appendix of the typhoid rabbits never demonstrated specific bacteria without positive findings in the duodenum and jejunum, while in several animals (rabbits 1353. 1416 and 1447) of exper. 8, paratyphoid bacilli could be demonstrated in the above mentioned portions of the intestinal tract without receiving their seedings from the upper segments or the bile. It is not unlikely that the constant involvement of the lymphatic structures of the intestines of paratyphoid rabbits leads to a discharge of specific bacteria into the intestinal content, producing the positive cultures recorded. Besredka [26] has recently called attention to this localization of Shiga dysentery and paratyphoid bacilli in the large intestines of rabbits and, based on these observations, he has developed an attractive theory on the local immunity of the intestinal mucosa to specific bacterial invasion. In connection with the discussion of our feeding experiments with organisms of the typhoid-paratyphoid group, this subject will be considered.

It has already been stated that the observations recorded in the foregoing were confirmed by several experiments with human paratyphoid strains. As distinct serologic differences have been demonstrated between these strains and true B. schottmülleri by numerous independent workers, and as it is as yet undecided whether the paratyphoid infection in man is identical with "rabbit typhoid," we repeated our immunity tests with several strains of B. schottmülleri isolated from typical human cases and sent to us by Mr. Harry Weiss of the Department of Bacteriology, Columbia University. Using a test inoculum (1-2 billion), which caused death after 6 to 8 days in the nonimmunized rabbits, the results were practically identical with those reported for the animal strains. An ability to destroy paratyphoid B bacilli more quickly is also evidenced in the immunized animals when a small number (300 million) of bacilli is intravenously inoculated; in fact, this phenomenon is particularly striking for the blood. To dispose of a number of organisms which can be destroyed by a normal animal in 5-6 days, an immune rabbit requires less than 24 hours. But in no instance was a cholecystitis produced: the number of bacilli injected was not sufficient even to cause an early discharge in the hepatic duct bile.

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[26] Ann. de l'Inst. Pasteur, 1919, 33, pp. 301 and 557.

In conclusion, it may be stated that the mechanism of destruction of the paratyphoid B bacilli when intravenously inoculated into immunized and normal rabbits differs from that constantly observed for the typhoid bacillus. This applies whether one uses B. aertryckei of rabbit origin or B. schottmülleri. Paratyphoid B. bacilli disappear more quickly from the tissues of the immunized than of the normal animals. It appears of interest in this connection to inquire into the nature of the factors responsible for this immunity, whether resident in the blood or tissues.

ATTEMPTS TO DETERMINE BY IN VITRO EXPERIMENTS THE FACTORS RESPONSIBLE FOR THE DESTRUCTION OF TYPHOID OR PARATYPHOID B. BACILLI IN THE RABBIT BODY

The original conception of Pfeiffer and his pupils, based on experiments primarily conducted on guinea-pigs, attributed the resistance to typhoid fever to bacteriolytic properties of the blood and lymph stream. This interpretation of the acquired immunity was challenged by Metchnikoff and his school, who believed that the leukocytes were mainly responsible for the destruction of the typhoid bacillus in the blood stream or the tissues of the immune animal or man. Based on the well-known experiments of Wassermann and Citron,[27] which demonstrated that antibodies may be produced by any cell or cell complex brought in contact with pathogenic bacteria, the theory of the local tissue immunity was proposed, but attracted little attention. However, in recent years the conception that in man the resistance to typhoid fever fever acquired during recovery from this disease is cellular and not humoral, has gained some encouraging experimental support. In this connection the studies of Wassermann and Sommerfeld [28] on mice, of Besredka [29] on rabbits, and of Liebermann and Acel [30] on guinea pigs, and the critical summary of Bessau,[31] which bridges the humoral and cellular theory in an attractive manner, should be mentioned. These studies are of great value from an experimental standpoint, but as we are unable to state whether the immunity mechanism of the guinea-pig, rabbit or mouse is identical with that in man, far reaching conclusions and analogy deductions are unquestionably premature and unjustified.

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[27] Deutsch. med. Wchnschr., 1905, 31, p. 573.

It is interesting to read in many publications dealing with the behavior of the typhoid bacillus in experimental animals, the statements that it is impossible to provoke in the usual laboratory animals, including the chimpanzees, a disease similar to typhoid fever in man, and that for these reasons the various factors and processes accompanying a human infection cannot be reproduced, nor can they be followed in the course of their intricate development. Yet the same writers usually untrained in comparative pathology, do not hesitate to regard certain facts elucidated, for example, on rabbits as indicative of a close analogy to the infection produced by the typhoid bacillus in man. It appeared to us that progress in the understanding of the mechanism of typhoid resistance could perhaps be made by analyzing our knowledge concerning the nature of the factors responsible for the native and acquired immunity of the rabbit against the typhoid or paratyphoid B bacillus. The in vivo experiments reported in the preceding paragraphs confirmed the observations of Bull, Arima, and Parker and Franke. Typhoid bacilli intravenously injected into rabbits are primarily destroyed in the liver, spleen, bonemarrow and lung. Even in the immunized rabbit the progress of the destruction of the deposited bacilli differs in no respect from that established for the normal animal. The test-tube experiments of Buxton,[32] Bull,[33] Teague and McWilliams,[34] and Stone showed, however, that the blood serum, plasma or defibrinated blood of immunized rabbits is nonbactericidal for the typhoid bacillus, while normal serum can destroy in 5-24 hours from 100,000-5,000,000 organisms. As numerous experiments conducted by us with the blood, its fluid and cellular elements produced results similar to those reported by these workers, it is considered unnecessary to present our additional data. It therefore may be surmised that the destruction of typhoid bacilli in the normal or unimmunized rabbits either cannot primarily be the result of bacteriolytic serum or lymph substances, or the in vitro experiments fail to reproduce the conditions as they exist in the rabbit body, a view held by Bail in 1905. The latter conception is apparently supported by some tests which we conducted with the serum of rabbits immunized against paratyphoid B bacilli.

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[28] Med. Klin., 1915, 11, p. 1307.

[29] Ann. de l'Inst. Pasteur, 1919, 33, pp. 301 and 557.

[30] Deutsch. med. Wchnschr., 1917, 43, p. 867; 1918, 44, p. 313; Biochem. Ztschr., 1918, 91, p. 46.

[31] Deutsch. med. Wchnschr., 1916, 42, p. 499.

[32] Jour. Med. Research, 1905, 13, pp. 305 & 431.

[33] Jour. Exper. Med., 1914, 20, p. 237.

[34] Jour. Immunology, 1917, 2, pp. 167, 185 and 375.

Exper. 10. Action of the Serum and Whole Blood of Normal and Immunized Rabbits in Vitro on Poratyphoid B Bacilli (B. Aertryckei).—The defibrinated blood or the fresh serum of normal healthy rabbits, or of those immunized by 3 subcutaneous injections with small doses of living paratyphoid B bacilli were tested for agglutinating value and for bactericidal power by means of the Buxton[32] or Teague and McWilliams[34] technic.

Thermolabile agglutinins for paratyphoid B bacilli in dilutions varying from 1:2-1:10 were demonstrated in the 4 rabbits tested for this purpose. The immune agglutinins of the protected rabbits reached frequently a titer of 1:10,000 and over.

One c c of fresh serum obtained from the heart blood of the normal and immunized rabbits was absolutely devoid of bactericidal properties for the B. aertryckei (rabbit paratyphoid B). Not even 5-15 organisms were destroyed in 24 hours. These serums in equal amounts were readily able to kill from 40,000-500,000 typhoid bacilli after an exposure of 24 hours. Similiar results were obtained with defibrinated blood. Human paratyphoid B bacilli reacted more irregularly; some rabbit serums would destroy from 100-1,000 bacteria. The human strains employed were about 2 years old; they had lost their invasive properties for the rabbit and in turn became more susceptible to the bactericidal powers of the blood.

These observations are not new. Citron,[35] Toepfer and Jaffe,[36] Neufeld and Hüne,[37] Werbitzki,[38] and recently Teague and McWilliams[34] have noted that the normal or immunized rabbit serum may possess no bacteriolytic substances while tropins can be demonstrated. In fact, Citron[35] and Tenbroeck[39] concluded from numerous experiments with the B. suipestifer that the presence or absence of serum antibodies does not permit conclusions relative to the degree of resistance of the rabbit against a subsequent infection.

[35] Ztschr. f. Hyg. u. Infektionskrankh., 1906, 53, p 515.

[36] Ibid., 1905, 52, p. 407.

[37] Arb. a. d. k. Gsndhsamte, 1907, 25, p. 164.

[38] Arch. f. Hyg., 1909, 70, p. 271.

[39] Jour. Exper. Med., 1917, 26, p. 441

The evidence thus far at our disposal suggests, therefore, that the destruction of typhoid as well as paratyphoid bacilli in the immunized rabbit cannot be explained by in vitro experiments with the whole blood or serum. Impressed by the conception of an histogenous immunity, so ably demonstrated for streptococcus infections in the cat, rabbit and guinea-pig (Hopkins and Parker, and Nagao),[40] and for staphylococcus infection in the dog (Bartlett, Ozaki [41] and Ito [42]) we were led to conduct numerous in vitro experiments with the tissues of infected normal and immunized rabbits. Typhoid and paratyphoid bacilli were used. One experiment is chosen to demonstrate the results obtained.

Exper. 11. Bactericidal Effect of the Tissues of the Normal and Immunized Rabbit Against B. Typhosus in Vitro and Vivo.—Two normal rabbits and 2 members of the same litter, which had been immunized with living typhoid bacilli according to Bull's method, were inoculated intravenously with 2,250,000 typhoid bacilli strain K. per 100 gm. of body weight. The average weight of the 4 rabbits was 2,860 gm. At stated intervals the animals were bled to death under ether. The organs were removed and distributed in sterile test tubes, which were placed in a water bath at 37 C. for from 2-5 hours. At the end of this time the pieces were weighed, ground and plated, as stated in previous experiments. The bactericidal effect of the leukocytes was tested by using clotted blood. The method given by Hopkins and Parker was followed. The results are summarized in table 9.

It will be noted from table 9 that at the end of the first 2 hours of incubation, the lung tissues of both the normal and immunized rabbits exerted in vitro a distinct bactericidal effect on the typhoid bacilli. There was a similar reduction in the spleen and bonemarrow, while in the liver, mesenteric lymph nodes, and kidneys the organisms multiplied moderately. Destruction by the excised tissue took place only during the first 2 or 3 hours of incubation, after which, owing probably to death of the cells, the organisms multiplied. On the other hand, in vivo the destruction progressed continuously and primarily marked in the lung and liver. At the end of the first 12 hours the lung tissue contained from $\frac{1}{30}$-$\frac{1}{50}$, and the liver from $\frac{1}{40}$-$\frac{1}{80}$ of the original number of typhoid bacilli deposited in these tissues. The spleen was less active, while the bonemarrow showed a distinct increase. The data pertaining to the clotted blood suggested that the leukocytes had exerted their supposed bactericidal effect. It should be stated, however, that the method of obtaining these results was not very accurate. In control

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[40] Jour. Infect. Dis., 1920, 27, p. 327.

[41] Jour. Med. Research, 1916, 35, p. 465.

[42] Ibid., 1917, 37, p. 189.

TABLE 9

Bactericidal Effect of the Tissues of the Normal and Immunized Rabbit Against B. Typhosus in Vitro and Vivo

Tissues	Rabbit 1287; Immunized and Agglutinated; 1:100,000; 6.3 Billion Injected			Rabbit 1287 (Normal 1:20+); 5.7 Billion Injected			Rabbit 1288 (Immune) (5,040,000,000)	Rabbit 1288B. (Normal) (6.3 Billion)
	10′ after Infection, Plated Immediately*	Plated after 2 Hours' Incubation*	Plated after 5 Hours' Incubation*	10′ After Injection, Plated Immediately*	Plated After 2 Hours' Incubation*	Plated After 5 Hours' Incubation*	Exsanguinated and Plated 12 Hours after Injection*	Exsanguinated and Plated 12 Hours after Injection*
Left and central liver........	300,000	980,000	2,650,000	900,000	810,000	3,000,000	8,000	5,000
Right and central liver......	....	....	....	....	....	....	4,800	6,800
Spleen......................	130,000	110,000	2,800,000	550,000	460,000	7,200,000	59,000	26,000
Bonemarrow................	62,000	25,000	800,000	21,000	15,000	22,000	225,000	88,000
Mesenteric lymph nodes.....	1,070	1,240	15,000	200	100	2,600	84	28
Lungs......................	70,000	30,000	700,000	20,000	10,000	60,000	1,200	740
Kidneys.....................	1,600	3,600	40,000	2,200	1,800	27,000	112	64
Urine.......................	0	....	....	0 (enriched +)	....	....	0	0
Femoral muscle.............	10	6	....	1	1	....	0	2
Gallbladder wall............	5,100	....	....	28,000	....	....	10	6
Bile........................	1	....	....	0	....	....	1	0
Carotis blood..............	(0.4 c c) 1,200 per c c	....	....	(0.7 c c, also E) 5,500 per c c	....	....	(0.8 c c) 1,500; 2,000; 1,000 per c c	(1.8 c c) 840; 760 per c c
Right ventricle..............	900 per c c	....	....	....	....	....	{ 1,860 } per c c	640 per c c
Left ventricle...............	300 per c c	....	....	1,400 per c c	....	....		
1 c c of blood clotted immediately..................	320	50	36	1,750	140	230; 300	....	....
Duodenal contents..........	13 colonies B. typhosus	....	....	Negative for B. typhosus	....	....	Negative for B. typhosus	Negative for B. typhosus

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* Number of colonies per 100 mg.

experiments a moderate multiplication took place, even in the first 3 hours of incubation. The solution of the question dealing with the typholytic effect of rabbits' leukocytes, in the light of the extensive investigations of Bail, Schneider and others, was sufficiently important to warrant a more detailed inquiry than we were able to conduct, at least for the present.

The degree of the destructive action of lung tissue in vitro is not constant. In some experiments it is decidedly more marked than in the one just reported. This statement is supported by the figures in table 10.

TABLE 10

COLONIES PER 100 MG. OF LUNG TISSUE

	Rabbit 1265B Typhoid	Rabbit 1265 Typhoid	Rabbit 1142 Typhoid Immunized	Rabbit 1412 Paratyphoid B Immunized	Rabbit 1481 Paratyphoid B Normal
Plated immediately............	14 400	110,000	226,000	20,800	3,800
Plated after 4 hours' incubation....	4,900	1,380	26,000	159,000	100,000

Observations made with paratyphoid B infected rabbits demonstrated a complete absence of bactericidal powers of the organs on incubation in vitro. Even the lung tissues were inactive. These facts are illustrated by the data presented in tables 8 and 10.

The results of the various experiments apparently established the fact that the organs of rabbits, with the exception perhaps of the lung, are unable to destroy typhoid or paratyphoid bacilli in vitro. The liver particularly, which in vivo is especially endowed to dispose of enormous numbers of bacteria, is inactive in the test tube. An explanation of this phenomenon is not possible, but it is certain that the bactericidal power of the rabbit organs is not only closely connected with a function of living protoplasm but is probably the result of an interaction between the blood and lymph substances and the tissue elements. Whether the polymorphonuclear leukocytes perform this function either alone or in conjunction with endothelial living cells and endothelial leukocytes (N. C. Foot),[43] together with certain blood plasma elements has not been determined. The histologic studies of Kyes,[44] of Hopkins and Parker,[45] of Bartlett, Ozaki and Ito[41] and of Nagao[40] with gram positive organisms, such as pneumococci, streptococci, staphylococci, respectively, and the observations of Schneider [46] with typhoid bacilli, indicate that both fixed and wandering phagocytes (macrophages) have the ability to engulf, as well as to digest, micro-organisms. The histologic studies of Mallory [47] and Gräff [48] established conclusively the fact that the typhoid bacillus produced in the reticulo-endothelial tissues of the lymph nodes, spleen, bonemarrow, liver, etc., a specific proliferation of the tissue histiocytes (Aschoff and Kiyono [49]). A casual study of the sections derived from the animals used in this report clearly demonstrated the remarkable phagocytic action of the endothelial cells of the liver and spleen and confirmed the well-known active function of the polymorphonuclear leukocytes. Several attempts to study the localization of the typhoid bacilli in their relation to the various cells have not been regularly successful. Even the best staining method (thionine, method of Zieler) produced indistinct and misleading pictures. Occasionally Kupffer's cells were found to be packed with bacilli when rabbits killed on the 6th hour were studied. Attention was therefore given to the general appearance of the various cells. Proliferation of the vascular endothelium in the spleen, liver, lung and lymph nodes of rabbits infected with B. aertryckei has already been mentioned by one of us in a previous communication, and it was pointed out on this occasion that these vascular reactions suggested a distinctive cellular mechanism of defense. The changes which can be noted in the sections were remarkably similar to those recently described by Nagao [40] for the streptococcus, and a detailed account of the data is contemplated in a paper of this series.

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[43] Ibid., 1919, 40, p. 353.

[44] Jour. Infect. Dis., 1916, 18, p. 277.

[45] Jour. Exper. Med., 1918, 27, p. 1.

The functions of the polymorphonuclear leukocytes as phagocytes, or as sources of typholytic substances, or as cells endowed with specific immunizing properties (Bachman [50]) cannot be denied since these properties can be demonstrated in vitro and vivo. The observations of Bull [7] on phagocytosis in vivo have been confirmed repeatedly. In thionine-stained smears made from the lung tissue, in preference to the liver and spleen pulp, of rabbits which had been inoculated with moderately large doses of B. typhosus and killed 10-60 minutes after the injection, we were able to count numerous polymorphonuclear leukocytes retaining intact and phagolyzed rods. In the paratyphoid B infected animals this phagocytosis occurred to a noticeable degree in the immunized, and to a lesser degree, in the normal rabbit. Whether this phagocytosis, however, only initiates the destructive mechanism cannot be investigated with certainty in vivo. We gained the impression that it was more or less a temporary process, but the subject will be considered more carefully in the next paper in connection with the analysis of the mechanism leading to the elimination of bacilli in the bile. Opportunity will be afforded to discuss the changes in the distribution of the polymorphonuclear leukocytes, which follow an intravenous injection in the first 5-120 minutes. Since the bacilli are taken up by the leukocytes it appears not at all unlikely that they are digested by a leuko-enzyme; this conception is supported by the demonstration of an ereptic enzyme in the rabbit's leukocyte by Parker and Franke.[51]

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[46] Arch. f. Hyg., 1909, 70, p. 41.

[47] Jour. Exper. Med., 1898, 3, p. 611.

[48] Deutsch. Arch. f. klin. Med., 1918, 125, p. 352; 126, p. 1.

[49] Folia haematol., 1913, 15, p. 383.

[50] Rev. de la Assoc. méd., Argentina, 1918, 29, p. 549; Rev. méd. del Rosario, 1919, 9, p. 1; Prensa méd., Argentina, 1919, 5, p. 253.

We desire to recall the observations of R. Schneider,[52] who showed by a set of ingenious experiments the active secretion of thermostabile bactericidal substances by leukocytes suspended in inactivated serum. This vital secretory function of the leukocytes is readily paralyzed by asphyxia of the cells with CO_2. The in vitro experiments conducted with rabbits' tissues by us and others have ignored this important fact. Is it not possible that the CO_2 content of the liver, spleen and bone-marrow in vitro inhibits the function of the leukocytes? It is, however, possible that the liver and spleen contained few leukocytes at the time the rabbit was exsanguinated, and the lung retaining the majority of these cells was particularly endowed to exhibit the described bactericidal properties. An investigation, with these conceptions in mind, is earnestly needed, as positive results would materially help to elucidate the many unexplainable impressions obtained through test-tube experiments. Attention should also be paid to the recent studies of Bachman [50] on a specific substance demonstrable in the polymorphonuclear leukocytes of immunized animals. This may, for example, prove that the leukocytes of rabbits immunized against paratyphosus B display a new property even in vitro, namely, a specific phagocytosis.

From this discussion it is quite evident that bactericidal substances directed against the typhoid and paratyphoid B bacilli are primarily of cellular origin, and are probably produced when needed at the places of bacterial invasion. In reviewing these facts one is once more reminded of the early studies of Wassermann and Citron [53] on local tissue immunity. According to the observations of these workers, it is proved that the tissues which come in intimate contact with the typhoid bacilli are capable of reacting locally by the production of specific bactericidal substances. For example, the cells lining the pleural cavity cannot only bind the specific antigen, but respond promptly by a production of bactericidal substances. Besredka [54] expresses a somewhat similar idea in his explanation of the intestinal immunity against paratyphoid-dysentery bacilli in the rabbit: "l'immunité naturelle vis à vis du virus typho-paratyphique, l'immunité acquise artificiellement repose également sur l'immunité de la paroi intestinale, ce qui veut dire qu'elle est locale."

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[51] Jour. Med. Research, 1919, 39, p. 301.

[52] Arch. f. Hyg., 1909, 70, p. 41.

[53] Deutsch. med. Wchnschr., 1905, 31, p. 573.

Some observations on the spleen discussed in the preceding paragraphs suggest that such a mobilization of bactericidal substances takes place in the organ itself. Ozaki [55] demonstrated that the accumulation of bacteria in the spleen was principally dependent on the vital activity of the cells, and Morris and Bullock [56] inferred from their extensive experiments that the spleen normally aids tremendously in resisting infectious processes in rats. These facts directed our attention to the spleen. It was constantly noted that in animals of the same litter the disappearance of the deposited typhoid bacilli was slow in the first 1-2 hours after the inoculation, irrespective of the marked local hyperleukocytosis and in contrast to the great reduction of their number in the liver and kidney. For example, in the first hour after the introduction of the infective dose, the spleen contained $\frac{1}{3}$ of the originally deposited bacilli, while at the end of the fourth hour, $\frac{1}{12}$; at the fifth, $\frac{1}{25}$; at the eighth, $\frac{1}{80}$; and at the end of the 24th hour, $\frac{1}{250}$ to $\frac{1}{400}$ of the original number was recordable in the spleen by the plating method. The figures dealing with the liver of the same animal were at the 8th hour, $\frac{1}{40}$; and at the end of the 24th hour, $\frac{1}{100}$ of the original number could be noted. These findings are subject to several explanations. From the recent studies of Teague and McWilliams,[57] it would seem that bacteriolytic substances could readily pass from the blood capillaries to the lymph, and by this route to the spleen and liver, which had been heavily invaded by typhoid bacilli. This hypothesis is applicable to an explanation of the destruction of typhoid bacilli in normal rabbits, but as these forces are not demonstrable in the typhoid and paratyphoid immunized rabbits, the conception of a cellular or a histohumoral immunity mechanism has far more justification. No doubt the suggestions of Manwaring and Coe,[58] dealing with the so-called endothelial opsonins, and the histologic studies of Nagao,[40] also deserve some attention in future experimental investigations.

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[54] Bull. de l'Inst. Pasteur, 1920, 18, p. 129.

[55] Jour. Med. Research, 1917, 36, p. 413; 37, p. 247.

[56] Ann. Surg., 1919, 70, p. 513.

[57] Jour. Immunology, 1917, 2, p. 375.

Additional observations illustrating phases of local immunity in the liver of immunized rabbits and guinea-pigs will be presented in paper 5. In connection with these experiments, it was repeatedly noted that the liver of rabbits which rapidly destroyed enormous numbers of typhoid bacilli were exceedingly voluminous, soft and heavy. For example, rabbits weighing from 2,000 to 2,200 gm. possessed, 4 to 8 hours after the inoculation, livers weighing 122 to 128 gm. These organs instead of representing $\frac{1}{30}$ had increased to $\frac{1}{16}$ of the body weight. A marked edematous infiltration characterized such livers histologically, and the condition must be interpreted as lymphostasis. We are not prepared to draw definite conclusions from this observation, but the well-known fact is recalled that rabbits' lymph obtained by stasis is strongly bacteriolytic for typhoid bacilli, and that the typholytic substances of such body fluids are of leukocytic origin (Schneider [52] and others).

The ability of excised pieces of lung tissue to destroy in vitro a large number of typhoid bacilli is apparently a fairly common function, having been demonstrated for streptococci and staphylococci. Moreover, it is certain that the perfused organ, freed from blood and, to a certain degree, of lymph, is capable of this action. Intercurrent infections, such as bronchopneumonia caused by B. bronchisepticus, seriously impair this destructive property. Moreover, extracts of the lung tissue are inactive. Whether some of the bactericidal substances (polymorphonuclear leukocytes) are preformed in the native immune rabbit, or whether they are mobilized locally, as in the paratyphoid B susceptible animal, is as yet unknown. According to Deutsch [59] the lung tissue of normal guinea-pigs always contains more agglutinins than the serum, while Briscoe [60] claims that phagocytosis by the pulmonary macrophages is more active and rapid when the animal has been previously immunized. The evidence thus far collected experimentally indicates, however, that the bactericidal substances are dependent on the action of the living cells. The entire mechanism of immunity in the lung is probably strictly cellular in origin and action, and is not influenced by a process of immunization. This definitely demonstrable histogenous bactericidal property, which is inherent in the lung is, however, of subordinate influence on the general mechanism of typhoid immunity in the rabbit. A careful experimental analysis of the pulmonary bactericidal powers will no doubt materially assist in disproving that humoral factors alone govern defensive and destructive immunity in typhoid fever and allied diseases.

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[58] Ibid., 1916, 1, p. 401.

[59] Ann. de l'Inst. Pasteur, 1899, 13, p. 689.

[60] Jour. Path. & Bacteriol., 1907-08, 12, p. 66.

THE NATURE OF IMMUNITY IN THE RABBIT AGAINST THE TYPHOID AND PARATYPHOID B BACILLUS

The studies of Bail,[61] of Hiss[62] and of Parker and Franke[51] indicate that the disease artificially produced in rabbits by the intravenous injection of typhoid bacilli is rather a toxemia than an infection ("ein Giftod"—Bail; "acute intoxication"—Hiss). Parker[63] has recently proved that the livers of very sick rabbits contain specific saline-extractable poisons. These toxins are of cellular origin and are the product of an interaction between the living cells of the liver, of other organs and the killed and disintegrated typhoid bacteria. Intoxication and death result primarily in those rabbits that possess a marked power to destroy bacteria in the liver and spleen. The susceptibility is therefore due to the bactericidal properties of its blood and tissues. Resistance to this intoxication is produced by active immunization with living typhoid bacilli, while typhoid immune serums failed to protect against the liver poison. We were able to confirm fully the observations of these workers. Rabbits capable of destroying in 1 c c of serum from 1,000,000 to 5,000,000 typhoid bacilli readily succumbed to the intoxication in from 5 to 48 hours, when injected with from 5 to 10 billion typhoid bacilli; and extractable liver poisons could be demonstrated in every instance. On the other hand, rabbits lacking typholytic substances in the blood or serum, and particularly immunized animals, would show symptoms of intoxication, although liver poison could not be found. The nonsusceptibility is either due to an absence of lytic antibodies or the result of a tolerance to the liver poison acquired in the course of the process of immunization. On the other hand, rabbits injected with paratyphoid B bacilli in moderate doses (less than 1,000 million) fail to show signs of intoxication for at least 48 hours; at the time of death their blood and organs contain enormous numbers of bacteria, but no poisons can be extracted from the liver. Paratyphoid infections in the susceptible rabbit exhibit all the signs of a septicemia. Instead of a progressive destruction of organisms in the blood and organs, as is shown for the typhoid rabbit, a progressive increase takes place. Resistance to this group of bacteria is developed by factors which prevent their multiplication. In vitro experiments indicate that this property is not in the blood serum, but confined to the living cells, and is only acquired by active immunization with living paratyphoid bacilli, a fact demonstrated by Citron [35] and TenBroeck.[39] The invasive B. aertryckei or the B. suipestifer produces a septicemia in the rabbit, while the typhoid bacillus causes a toxemia. It should, however, be borne in mind that only very large doses of living typhoid bacilli are capable of causing this intoxication in susceptible rabbits, while even small doses of paratyphoid B bacilli produce a fatal septicemia. Moreover, the defensive mechanism against the intoxication, which saves the life of the animal, is called on during the first 10 hours after the injection of the typhoid bacillus, while in the paratyphoid infection a similar mechanism may play a rôle only toward the end of the disease. It is, however, well known that typhoid rabbits may die from a moderately large initial injection on the 3rd to the 10th day. In these instances we have failed to find extractable liver poisons, but have demonstrated enormous numbers of bacteria in the bonemarrow, a moderate number in the spleen and liver, and few or no organisms in the blood. For illustration of this point the reader is referred to rabbit 1686 in table 6. Our observations unquestionably corroborate the conclusions of Parker and Franke that in rabbits which are slowly dying of typhoid the bonemarrow may constitute a focus of infection from which the organisms are continually swept into the blood stream and from there taken up by the organs. The actual mode of this localization, whether in the epiphysis marrow or elsewhere, is of great interest but by no means clearly understood (I. Koch [64]). Comparative leukocyte counts by Studer,[65] C. W. Wells [66] and our own indicate that the individual response of the bone-marrow of rabbits, following the injection of typhoid bacilli may vary considerably. In a certain percentage of animals serious disturbances in the leukocytogenic functions follow the injection of typhoid, paratyphoid and coli bacilli, which in turn lead to an exhaustion of the leukocytes and to a breakdown of the defensive

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[61] Arch. f. Hyg., 1905, 52, p. 272; Wien. klin. Wchnschr., 1907, 20, p. 275.

[62] Jour. Med. Research, 1913, 28, p. 389.

[63] Jour. Exper. Med., 1918, 28, p. 571.

[64] Ztschr. f. Hyg. u. Infektionskrankh., 1911, 69, p. 436.

[65] Thesis, Univ. of Zürich, 1903.

[66] Jour. Infect. Dis., 1917, 20, p. 219; 1918, 22, p. 502.

mechanism of the body. Future studies should attempt to correlate these observations. It is, however, not unlikely that the numerous infarcts of the liver and spleen going on to necroses as the result of typhoid toxemia constitute also seed beds for the invasion of the blood stream. These toxic necroses lack proper lymph circulation, according to Gräff [48] and favor, as has been demonstrated by Benian,[67] the development of immune body-fast, parasitic typhoid bacilli. Furthermore, the protection of pathogenic organisms by living, endothelial cells, shown by Rous and Jones,[68] must be kept in mind. Unhindered development of the bacteria in the gallbladder constitutes, in our experience, only in exceptional instances a focus responsible for the infection of the blood stream, as will be demonstrated in paper 5, on the mechanism of gallbladder infection.

Continuous invasion of the blood stream and removal of the organisms by the viscera is accompanied, in the first place, by an accumulation of typhoid bacilli in the liver and in the spleen. In these tissues the main destruction takes place; the liberation of toxic albumoses by disintegration, or the production of toxic substances by some form of proteolysis, unquestionably produces a profound injury, which can be seen macroscopically. The inability to demonstrate poison in the liver is probably due to the fact that its liberation is gradual and in amounts which do not exceed the detoxicating threshold of the organs. Yet this detoxicating function, which probably is present in other organs than the liver, continues as long as bacteria are swept into the blood from the various foci mentioned; but the prolonged production of toxins may seriously damage the metabolic function of the organs, causing cachexia and the death of the animal. Immunized rabbits, which succumb to very large injections of typhoid bacilli, present the same cultural findings (of a low blood and a high bonemarrow count). It appears, therefore, not unlikely that aside from the tolerance against specific poisons, immunity against the typhoid bacillus in rabbits is represented by their ability to prevent the formation of bonemarrow and visceral foci.

In rabbit paratyphoid the distribution of bacteria in the blood and organs is quite uniform shortly before death on the 6th or 7th day, irrespective of the original route of infection. The blood contains large numbers of bacteria per c c, and the tissues a proportional share, according to size. The septicemia is probably the consequence of the washing out of organisms from the foci of active multiplication. The enlarged spleen, in case the gallbladder is not infected, is the chief source; while the bonemarrow, liver and lungs are less important. Liver poisons cannot be demonstrated for the reasons above outlined, but the anatomic lesions and the clinical symptoms suggest that the death of the animal is probably the result of an intoxication. Immunity against a paratyphoid B infection consists, therefore, in an ability to prevent the invasion and multiplication of the bacteria, and with it the development of poisons which injure the function of the important organs. This immunity mechanism is effective following the subcutaneous inoculation of living bacteria, but it can be readily overcome by the intravenous inoculation of moderately large doses of paratyphoid B bacilli. Overwhelming blood infections of the immunized rabbit produce a cycle of events identical to that described for the typhoid animals. The intoxications provoked by the inoculation of heat-killed paratyphoid bacilli or their extracts have not been investigated, and therefore no attempt is made to explain the mechanism of susceptibility and nonsusceptibility to preformed toxins.

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[67] Jour. Path. & Bacteriol., 1920, 23, p. 171.

[68] Jour. Exper. Med., 1916, 23, p. 601.

From this discussion it is evident that the disease produced in the rabbit by intravenous injection of typhoid or paratyphoid B bacilli must be considered a specific toxicosis. In this term we include the rôle played by so-called endotoxins, and assume in addition that toxic substances (perhaps amines) are produced from the host's protein by some form of proteolysis, which is stimulated by the disintegrated bacteria and their products. The abrupt increase in the nitrogenous excretion (primarily the allantoin fraction) of rabbits injected with living typhoid bacilli, demonstrated by Pepper and Miller,[69] is no doubt a distinct indication of an extensive katabolism of body protein. Resistance to this form of experimental disease is a local or general "antitoxic adaptation immunity" and cellular in nature. The native immunity of rabbits for the typhoid bacillus depends entirely on (1) the ability of the endothelial cells, polymorphonuclear leukocytes, and perhaps the body fluid to destroy the invading bacteria; (2) the mechanism to prevent the formation of foci from which the blood stream is constantly seeded; and (3) the detoxicating power of the liver and the tissues in general. These factors, inherent in the rabbit, can be made inoperative by the injection of a very large dose (over 5,000 million) of typhoid bacilli. An acquired immunity by means of dead or living vaccines can even then prevent the final issue produced by the exhaustion of the bonemarrow with its resulting toxemia. Such an acquired resistance is primarily cellular and mainly directed against the liberated toxins. It appears that the actual destruction of the bacteria and the elimination of foci responsible for the continuous invasion of the blood stream is managed by the same, perhaps slightly enhanced, cellular factors responsible for the inherent native immunity.

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[69] Jour. Infect. Dis., 1916, 19, p. 694.

Susceptibility of the rabbit to paratyphoid B bacilli resolved itself into an inability of certain tissues and their cells, perhaps together with the blood, to kill the introduced bacteria completely. As a result, foci occur in the least resistant tissues (bonemarrow and lymphatic tissues), and the blood stream and organs are gradually invaded with newly developed bacteria. Organs like the liver and spleen, which were able to free themselves to a large extent of living paratyphoid bacilli during the first period of the infection, are then overwhelmed with bacteria and their toxins. Immunity produced by recovery from a slight infection consists of a fairly speedy sterilization of the tissues and blood, together with the elimination of foci, which act as seed beds for the ensuing septicemia in susceptible animals. This is probably not the same mechanism as that evolved by immunization with dead bacteria.

Various questions suggest themselves; for example, Is the immunity produced in rabbits against typhoid or paratyphoid bacilli comparable with the one provoked in man by the prophylactic inoculation of vaccines? From the information at hand it is impossible to answer this important question. Our experiments on guinea-pigs, cats, and monkeys indicate that each species disposes of the intravenously injected organisms of the typhoid-paratyphoid group in its own characteristic way. The immunized guinea-pig destroys typhoid and paratyphoid bacilli more readily than the normal; while monkeys and cats behave like the rabbit. It is quite possible that typhoid fever in man really is a toxicosis, or an endotoxin disease, as proposed by Staddelmann and Wolf-Eisner.[70] The immunity mechanism could then follow either the course outlined for the typhoid or the paratyphoid B infection in the rabbit. It may, however, be entirely different and be more readily comparable with the conditions found in the guinea-pig. The available bacteriologic and immunologic data pertaining to typhoid fever in man are not sufficiently complete to venture these far-reaching deductions based on analogy.

[70] München med. Wchnschr., 1907, 54, pp. 1161 and 1237.

CONCLUSIONS

A small number of typhoid bacilli inoculated intravenously into normal rabbits of the same litter was rapidly removed from the blood stream at the end of from 10 to 15 minutes after the inoculation. About 20 to 30% of the inoculum were found in the liver; a smaller amount in the spleen, bonemarrow and lungs; and comparatively small numbers in the lymph nodes, kidneys, muscle, etc.

The gallbladder bile contained only a small number of bacteria, while the gallbladder wall received, according to its size, a proportional share of the total number of bacteria deposited in the liver. The intraportal injection presented no advantage over the common intravenous method.

Large numbers of typhoid bacilli are less speedily removed from the blood stream, but the distribution in the organs remains the same in the first 4 to 8 hours.

The bacteria taken up by the tissues are rapidly reduced in number, particularly in the liver, spleen and lungs. The bonemarrow is less active and not infrequently the typhoid bacilli multiply in these tissues and form foci, which reinfect the blood stream. The gallbladder can become infected either as a result of an immediate extensive elimination of the introduced bacteria, or through the continuous discharge of bacilli from liver foci in the hepatic bile, or in consequence of an embolic infarction of the gallbladder capillaries causing a diphtheritic cholecystitis. In the latter instance the cystic bile may not become invaded with typhoid bacilli for 24-48 hours after the intravenous injection.

The distribution and destruction of small or large doses of typhoid bacilli is practically the same in the normal as in the immunized rabbit, whether protected by the injection of dead or living organisms. The only noteworthy difference between the normal and immunized rabbit is found in the lung and spleen, namely, the lung of the immunized rabbit takes up a somewhat greater number of bacteria than the normal; and the spleen of the immune sterilizes itself more slowly than the normal.

Localization in the gallbladder and bile 96 to 120 hours after the infection occurs more readily with moderately heavy suspensions prepared from rabbit-blood agar than those obtained from peptic-digest agar slants.

Rabbits which succumb to the typhoid intoxication regularly harbor enormous numbers of typhoid bacilli in the bonemarrow of the long bones; these foci are probably the seed beds responsible for the continuous invasion of the blood stream and the subsequent overwhelming infection of the liver and spleen.

Paratyphoid A bacilli act in a manner identical to typhoid bacilli. The mechanism of destruction of paratyphoid B bacilli, whether B. aertryckei of rabbit origin, or B. schottmülleri in the immunized and normal rabbit, is different from that reported in the foregoing for the typhoid bacillus. As a rule, paratyphoid bacilli disappear more quickly from the blood and tissues of immunized than normal animals. The activity of the splenic tissue in the destruction of these bacteria is noteworthy and deserves further investigation.

The blood and serum of normal rabbits in vitro is bacteriolytic for typhoid and paratyphoid A bacilli, but is inactive for paratyphoid B bacilli. The serum of immunized rabbits in vitro, whether protected against typhoid, paratyphoid A or paratyphoid B bacilli, is nonbactericidal.

Pieces of excised organs of exsanguinated rabbits heavily infected with bacilli of the typhoid-paratyphoid group when incubated in test tubes at 37 C. may show in the first 2 hours a slight bactericidal action. The property is marked in the excised lung tissues, but not in its extracts and is dependent on the living protoplasm of the cell.

The nature of the immunity of the rabbit against the typhoid and paratyphoid B bacilli is considered in some detail, and it is pointed out that an intravenous injection of a large dose of B. typhosus produces primarily a toxemia, while the invasive B. aertryckei provokes in small amounts a progressive septicemia. The various cellular factors, which, in the light of available knowledge, are responsible in the normal and immunized rabbit for the local and general antitoxic adaptation immunity are discussed.

XXIV

THE MECHANISM OF GALLBLADDER INFECTIONS IN LABORATORY ANIMALS

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. V

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Experimental gallbladder infections have been produced in the rabbit, guinea-pig, etc., by numerous investigators, following intravenous injections of typhoid, paratyphoid, dysentery and coli bacilli, streptococci, staphylococci and even nonpathogenic organisms. A perusal of the numerous publications indicates that these infections of the biliary system are, however, not developed with any degree of certainty. In spite of the excellent experimental studies of Doerr,[1] Blumenthal,[2] Else,[3] Nichols [4] and others, our knowledge is still incomplete concerning the exact mechanism which provokes in the rabbit a localization of the inoculated typhoid bacilli. Moreover, it has been customary to look on the gallbladder as the only great breeding place of typhoid bacilli, whenever they have been found in this viscus a few weeks after the inoculation of the infective dose. No doubt there is a considerable accumulation of data pointing in this direction, but there are observations on rabbits and guinea-pigs, namely, those of Hailer and his associates,[5] and Emmerich and Wagner,[6] which do not conform to this conception of the carrier state. These workers have demonstrated that in about 10% of instances the cystic bile may be found sterile, although the wall contains typhoid bacilli. The biliary passages may likewise be sterile and yet the liver (in 80% of the instances), the bone-marrow (in 25 to 50% of the instances) and the spleen (in 38% of the instances) may harbor the bacilli for more than 30 days. Moreover, the majority of experimental pathologists who study the gallbladder carrier state in rabbits, overlook the early observation of

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[1] Centralbl. f. Bakteriol., I., 1905, 39, p. 624.

[2] Ibid., 1910, 55, p. 341.

[3] Surg., Gynec. & Obst., 1910, 11, p. 470.

[4] Jour. Exper. Med., 1914, 20, p. 573; 1916, 24, p. 497; 1917, 68, p. 958.

[5] Deutsch. med. Wchnschr., 1912, 38, p. 2267; Arb. a. d. k. Gsndhtsamte, 1914, 47, pp. 303, 451, and 470. Hailer, E., and Wolf, G.: Arb. a. d. k. Gsndhtsamte, 1914-15, 48, p. 80.

[6] Centralbl. f. allg. Path. u. path. Anat., 1916, 27, p. 433.

Blachstein;[7] namely, in chronic or subacute infections of the gallbladder contents the lesions from which the infection occurs exist in the liver in the form of necrotic or inflammatory foci. Keeping these facts in mind, it seems advisable to regard the biliary tract as an excretory channel and the cholecystitis more as a sequel than as the source of the continual and repeated infection of the bile. A similar but novel view has recently been advanced by Webb-Johnson[8] to explain the human intestinal carrier state. This writer assumes the spleen to be the primary focus responsible for the elimination of typhoid bacilli in the bile. Opportunity will be afforded to discuss these conceptions in the course of the analysis of our experimental data.

Following an intravenous injection of typhoid bacilli, such organisms can be demonstrated quite regularly in the gallbladder, but the duration of their sojourn is exceedingly variable and in many instances limited to a short period. A study of the pathogenesis of the experimental carrier state in laboratory animals must therefore consider (a) the mechanism by which the bacilli reach the gallbladder and (b) the concomitant factors in the form of anatomic or physiologic changes, which either lead to a prolonged persistence or to a rapid disappearance of the invading typhoid bacilli. Among these changes may be mentioned: the pathologic processes in the gallbladder wall (cholecystitis and cholangitis); peculiarities in the physiology of the bile with reference to its secretion, composition and reaction; and abnormalities in the biliary system leading to the formation of gallstones. Consideration must also be given to the question of the individual immunity of the infected animal. It is the purpose of this paper to investigate these contributory factors in connection with their bearing on the mechanism of gallbladder infections.

GENERAL CONSIDERATIONS

It is generally stated that theoretically bacteria may reach the gallbladder in the following manner: (a) direct infection (puncture); (b) ascending infection from the intestines via ductus choledochus; (c) descending infection (with the bile secreted from the liver); (d) arterial embolus of the wall vessels; (e) through the lymphatic and venous blood vessels. Each of these possibilities has been investigated experimentally and has been shown to occur in typhoid cholecystitis in the rabbit. Some of the observations have even *sine conditione* been applied to explain human cholecystitis, and the intestinal carrier state. A brief consideration is therefore justifiable to determine, if possible, which of these routes is the one usually followed in the experimental reproduction of gallbladder infection in animals and to what extent the findings are analogous to those present in the human carrier state.

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[7] Bull. Johns Hopkins Hosp., 1891, 2, pp. 96 and 121.

[8] The Lancet, 1917, 2, p. 813; Surgical Aspects of Typhoid and Paratyphoid Fevers, London, 1919, p. 167.

Infection through trauma (puncture) occurs in rare instances only as a result of surgical procedures or manipulations. The direct inoculation of various bacteria into the gallbladder of rabbits and guinea-pigs has been practiced experimentally by Gilbert, and Dominici[9] and Fournier,[10] Talma, Richardson,[11] Italia,[12] Ehret and Stolz,[13] Forster,[14] Violle,[15] Marxer,[16] Uhlenhuth and Messerschmidt,[17] Klinkert,[18] Hailer and Ungermann,[19] Lange and Roos,[20] Schöbl,[21] Emmerich and Wagner,[22] and Venema.[23] The published reports indicate that this method produces with greater regularity a localization of the bacteria in the gallbladder than the intravenous method of infection. Constant results with the typhoid bacillus can be expected in rabbits, according to Hailer and Ungermann, only until the 30th day after the operation. In a few animals the bacilli disappear at the end of this period, while in others a chronic inflammation favors their persistence for from 217 (Hailer and Rimpau) to 341 days (Emmerich and Wagner). The results which are obtained with the typhoid bacillus in guinea-pigs are less satisfactory (Marmoreck.[24] Wagner and Emmerich,[25] Thompson and Meyer[26]). The gallbladder infections provoked in rabbits by intracystic infections of cholera vibrios or dysentery bacilli (Schöbl,[21] Nichols,[27] Meyer and Stickel [28]) are characterized by a benign process, which shows a tendency to rapid recovery. The organisms disappear from the injured viscus at the end of from the 15th to the 30th day. All authors, however, agree that direct inoculation of the gallbladder is the route par excellence for the production of experimental gallbladder infections.

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[9] Bull. d. l. Soc. de biol., 1893, 5, p. 1033.

[10] Compt. rend. Soc. de biol., 1897, 49, pp. 692 and 636.

[11] Boston Soc. Med. Sc., 1898-99, 3, p. 79.

[12] Policlin. Roma, 1901, 8.—C., p. 153.

[13] Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 350; 1900, 7, p. 372; and 1901, 8, p. 153.

[14] Forster, in Uhlenhuth, P., and Messerschmidt, T.: Deutsch. med. Wchnschr., 1912, 51, p. 2367.

[15] Ann. de l'Inst. Pasteur, 1908, 22, p. 341.

[16] Ztschr. f. Chemotherap., 1914, 2, p. 23.

[17] Deutsch. med. Wchnschr., 1912, 51, p. 2367.

[18] Berl. klin. Wchnschr., 1911, 48, p. 335.

[19] Deutsch. med. Wchnschr., 1912, 38, p. 2267; Arb. a. d. k. Gsndhsamte, 1914, 47, pp. 303, 451 and 470.

[20] Ibid., 1915, 50, p. 57.

[21] Jour. Infect. Dis., 1916, 18, p. 307; 19, p. 145; Philippine Jour. Trop. Med., 1916, 11, p. 153; 1917, 12, p. 23.

[22] Med. Klin., 1916, 12, p. 74; Zschr. f. Immunitatsforsch. u. exper. Therapie, 1916, 24, p. 557; Centralbl. f. allg. Path. u. path. Anat., 1916, 27, p. 433.

[23] Berl. klin. Wchnschr., 1917, 54, p. 815.

[24] Med. Klin., 1916, 12, p. 275.

[25] Centralbl. f. Bakteriol, 1916, 79, p. 1.

[26] To be published.

It is obvious that the foregoing method does not explain the route by which the typhoid bacillus reaches the gallbladder in the course of the disease. The early finding of typhoid bacilli in the gallbladder of fatal typhoid cases by Gilbert and Girode, Chiari,[30] Pratt, and others, in view of the conception (prevailing at the time of their observations) that typhoid was a purely intestinal disease, was explained by the assumption that the bacillus reached the gallbladder by an ascending route from the intestines through the ductus choledochus. Unquestionably the ingenious experiments of Homén,[31] Ehret and Stolz, Netter, Naunyn, Doerr, Blumenthal and others, which demonstrated the occurrence of bacteria in the bile subsequent to the ligation of the common duct, contributed greatly to the abandonment of this theory as it pertains to the typhoid bacillus. We have been unable, in a number of experiments, to produce gallbladder infections by the introduction of enormous doses of typhoid bacilli into the duodenum close to the papilla of Vater. These results are analogous to those of Hailer and Ungermann. It is needless to state that these findings apply only to the typhoid bacillus, because in the light of numerous clinical and pathologic observations it seems reasonable to suspect an occasional invasion of bacteria or protozoa by the ascending route. Such a mode of infection is particularly favored by intestinal or biliary stasis. The invasion probably never occurs through the common duct against the bile current except in infections with the entameba, but follows the lymphatics of the duct, as has been definitely demonstrated in ascending renal infections; B. coli, B. dysenteriae and Cholera vibrio infections may be mentioned as possible examples of this type of gallbladder infection. In fact, Schöbl [32] has demonstrated experimentally in some guinea-pigs the presence of cholera vibrios in the bile 7 to 14 days after the feeding of the bacteria.

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[27] Jour. Exper. Med., 1916, 24, p. 497.

[28] To be published.

[30] Verbandl. d. Deutsch. path. Gesellschaft, 1907; Erganz. heft. z. Centralbl. f. allg. Path. u. path. Anat., 1908, 18, p. 143.

[31] Ibid., 1894, 5, p. 825.

[32] J. Infect. Dis., 1916, 18, p. 307.

Descending or so-called hemato-hepatogeneous infection of the bile has been repeatedly verified experimentally and is now generally accepted. Birch-Hirchfeld[33] was the first to state that micro-organisms reach the gallbladder through the circulation either by way of the entero-hepatic blood stream (vena portae) or the arteria hepatica. It is a proved fact that various micro-organisms inoculated intravenously or into a radical of the portal vein of rabbits, dogs, guinea-pigs, and other animals appear in from 2 to 50 minutes in the bile collected from temporary common duct or permanent gallbladder fistulas or from the cystis bile at necropsy. The early studies of v. Fütterer [34] in 1888-1899 on fistular animals have been verified by Biedl and Kraus [35] and by Nichols.[27] Pernice and Scagliosi,[36] Sherrington,[37] Pawlowsky,[38] Métin,[39] Heck [40] and others have inoculated animals subcutaneously, intraperitoneally, or intravenously with various organisms and have tested after varying time intervals the bile or urine for the presence of the inoculated bacteria. The results have been irregular. For example, Sherrington reports that after the injection of anthrax bacilli, staphylococci and pyocyaneus only 18 biles of a series of 49 proved to be infected, while Heck and Métin record negative results. Similarly disappointing have been the experiments of Carmichael [41] and Else,[3] who introduced the bacteria by way of the portal vein. On the other hand, it is stated by Breton, Bruyant and Mezie,[42] that even the introduction of B. prodigiosus by gavage produces in a guinea-pig with a ligated common duct an invasion of the gallbladder bile 3 to 4 hours after the introduction of the bacteria. In our feeding experiments with typhoid bacilli, we have cultivated the bacilli occasionally from the liver, but never from the bile. Even in the course of a successful series of paratyphoid feeding infections, we have not succeeded in demonstrating the bacteria in the bile, although as a rule they have been abundant in the liver (Litch and Meyer[43]). This definitely proves that the presence of micro-organisms in the liver is not necessarily followed by an invasion of the bile.

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[33] Lehrbuch d. path. Anat., Ed. 4, 1895, 2, p. 694.

[34] Berl. klin. Wchnschr., 1899, 36, p. 58; Medicine, 1895, 1, p. 279.

[35] Ztschr. f. Hyg. u. Infektionskrankh., 1897, 26, p. 353; and Zentralbl. f. inn. Med., 1896, 17, p. 737.

[36] Deutsch. med. Wchnschr., 1892, 18, p. 761.

[37] Jour. Path. and Bacteriol., 1892-93, 1, p. 259.

[38] Ztschr. f. Hyg. u. Infektionskrankh., 1900, 33, p. 261.

[39] Ann. d. l'Inst. Pasteur, 1900, 14, p. 415.

[40] Ztschr. f. Hyg. u. Infektionskr., 1907, 56, p. 1.

[41] Jour. Path. and Bacteriol., 1903, 8, p. 276.

[42] Comp. rend. Soc. de biol., 1912, 72, p. 13.

Since Blachstein[7] found that rabbits, which have been given intravenous injections of atoxic strains of B. coli or B. typhosus, yield cultures from the bile for many days or weeks subsequently, numerous workers have conducted similar experiments. The observations on rabbits by Miyake,[44] Adrian,[45] Doerr,[1] Forster,[46] Lemiérre and Abraham,[47] Chiarolanza,[48] Conradi,[49] E. Blumenthal,[2] Morgan,[50] Tanabe and Takeuchi,[51] Arima,[52] Bully,[53] Perussia,[54] Johnston,[55] Hailer and Rimpau,[5] Gay and Claypole,[56] Nichols,[57] Weinfurther,[58] Gibson,[59] Lentz, Hailer and Wolf,[60] Besredka,[61] Flu,[62] and others with typhoid-paratyphoid and dysentery bacili; Baroni and Ceaparu,[63] Cano,[64] Schöbl,[21] and Creig,[65] with Vibrio cholerae; J. Koch[66] with staphylococci and streptococci; and Rosenow,[67] Ophüls and Smith,[68] with streptococci, indicate that intravenously inoculated bacteria must reach the gallbladder quite frequently and produce a distinct cholecystitis. It even has been demonstrated by Fränkel and Much,[69] and by

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[43] Jour. Infect. Dis., 1921, 28, p. 27.

[44] Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 479.

[45] Ibid., 1901, 7, p. 407.

[46] München. med. Wchnschr., 1905, 52, p. 1473.

[47] Compt. rend. Soc. de biol., 1907, 63, p. 252.

[48] Ztschr. f. Hyg. u. Infektionskrankh., 1909, 62, p. 11.

[49] Ztschr. f. Immunitätsforsch. u. exper. Therap., 1910, 7, p. 158.

[50] Jour. Hygiene, 1911, 11, p. 202.

[51] Mitt. a. d. Mediz. Gesellsch. z. Osaka, 1910, 9, refer.; Centralbl. f. Bakteriol. I. Ref., 1911, 50, p. 294.

[52] Arch. f. Hyg., 1911, 73, p. 265; Centralbl. f. Bakeriol., I, 1912, 63, p. 424.

[53] Ztschr. f. Hyg. u. Infektionskrankh., 1911, 69, p. 29.

[54] Pathologica, 1912, 4, p. 141.

[55] Jour. Med. Res., 1917, 37, p. 189.

[56] Arch. Int. Med., 1913, 12, p. 616.

[57] Jour. Exper. Med., 1914, 20, p. 573, 1916, 24, p. 497.

[58] Centralbl. f. Bakteriol., 1914-15, 75, p. 379.

[59] Jour. Roy. Army Med. Corps, London, 1917, 29, p. 601.

[60] Arb. a. d. Reichs Gsndhsamte, 1918, 51, p. 1.

[61] Ann. de l'Inst. Pasteur, 1919, 33, pp. 557 and 301.

[62] Geneesk. Tijdschr. v. Nederl. Indie, 1918, 58, p. 67.

[63] Compt. rend. Soc. de biol., 1912, 72, p. 894.

[64] Centralbl. f. Bakteriol., 1913, 72, p. 124.

[65] Indian Jour. Med. Res., 1913-14, 1, pp. 44 and 59; 1914-15, 2, pp. 1, 28, and 907; 1915-16, 3, pp. 259 and 397; 1917, 4, pp. 651 and 658; 1917-18, 5. pp. 81 and 89.

[66] Ztschr. f. Hyg. u. Infektionskrankh., 1908, 60, p. 335; 1908-09, 62, p. 1; 1911, 69, p. 436.

[67] Jour. Infect. Dis., 1916, 14, p. 527.

[68] Proc. Soc Exper. Biol. and Med., 1918, 15, p. 113.

[69] Ztschr. f. Hyg. u. Infektionskrankh., 1911, 69, p. 342.

E. Fränkel,[70] that paratyphoid B or A bacilli may exhibit selective properties whereby they tend to realize in the gallbladder and bile of animals infected by intraperitoneal injections or by feeding. Most of the workers mentioned agree that the bacteria reach the gallbladder in the bile secreted by the liver. In some instances (F. Blumenthal and Nichols) they may arrive in from 5 to 10 minutes. The bile capillaries apparently receive the organisms from the blood of the interlobular veins, the endothelial lining being the only barrier which separates the biliary system from the blood stream. The following questions naturally arise: 1. Is this intrahepatic passage of micro-organisms from the blood to the bile a normal secretory function? 2. Is it a mechanical process? 3. Does this elimination follow the pathologic changes which have been produced in the blood vessel wall by the bacterial toxins? Neither Sherrington nor J. Koch, both careful workers, believe in a true physiologic elimination, but they agree that a transit of bacteria across the hepatic membranes can occur without the detectable presence of blood in the infected bile. It is furthermore stated by Sherrington that the membrane may remain normal, unruptured and impervious to blood cells, while the soluble toxin of the bacteria may injure the barrier sufficiently to cause a slight inflammation which is followed by the passage of the accumulated micro-organisms. His view is well supported by the following facts: First, in his experiments nonpathogenic bacteria never appeared in the bile. Second, J. Koch,[71] failed to produce cholecystitis with avirulent skin staphylococci, injected in small doses. Furthermore, Wyssokowitch,[72] and also Blachstein considered the liver necroses prerequisites for the infection of the biliary secretions. It has been pointed out in the preceding paper (IV), confirming the observations of Weinfurter and Nichols, that the intravenous injection of typhoid bacilli produces bile invasion or infection only when large doses of recently isolated strains are employed. Dosage, virulence and lesions and not the secretory, detoxifying activity of the liver. are the prerequisites for the passage of bacteria from the blood to the bile capillaries. In the light of these established facts the conception of a purely mechanical passage deserves little consideration. It is most unlikely that the masses of bacteria, which are thrown into the liver capillaries, cause "permeability of the liver filter," by rupturing the

[70] München. med. Wchnschr., 1918, 65, p. 413.

[71] Ztschr. f. Hyg. u. Infektionskrankh., 1908, 60, p. 335.

[72] Ibid., 1886, 1, p. 3.

capillary wall and by being flushed with the liver secretion into the biliary capillaries. Our experiments, to be reported later, indicate that the transit of bacteria from the blood to the bile is governed by the endothelial lining of the blood capillaries.

The analysis of the various routes by which bacteria reach the gallbladder does not exhaust all the possibilities. Particularly in connection with the typhoid gallbladder problem, there has arisen a controversy of considerable interest. The descending hemato-hepatogenous passage of typhoid bacilli, as described in the foregoing, was accepted as the most likely mode of bile invasion until J. Koch and Chiarolanza [48] claimed to have proved that the bile may also become infected through the capillaries of the gallbladder wall. Koch drew his conclusion from the histologic picture in a human case of typhoid cholecystitis, in which he found nests of typhoid bacilli lying in close relation to the capillaries of the folds of the mucous membrane. Lange and Roos,[20] Creig,[73] and others (Posselt [74]) have more or less accepted this view. At Koch's suggestion Chiarolanza tied the cystic duct of rabbits in two places, and injected immediately following the operation typhoid bacilli intravenously. He recovered them from the gallbladder 24 to 48 hours later. These experiments have been justly criticized by E. Blumenthal and Nichols. A careful scrutiny of the protocols published by Chiarolanza indicates that the bile was bloody even in animals examined in less than 24 hours. Technically, the experiments have been poorly executed; he admits that in the majority of incidences the entire gallbladder has been necrotic. The illustration in Figs. 6 and 7 of his article, which show bacterial emboli in the wall, supply ample proof for this statement. In ligating the cystic duct the accompanying cystic artery was in all probability also tied and hemorrhagic infarction occurred from incomplete collateral circulation.

Those who attempt to refute the general conception of an embolic wall infection, depend for their argument on certain experiments conducted by R. Doerr.[1] This worker also tied the cystic duct, but waited from 3 to 5 days before giving the intravenous injection. Under these conditions no infection occurred. The conclusions drawn from these experiments are for the following reasons invalidated: First, no cultures were made of the gallbladder wall in which a focus of infection may be present from 24 to 72 hours before the bacteria break through the epithelium leading to contamination of the cystic bile. Second, only one experiment is reported, and in this no mention is made of the number of animals in which either one-half or one loopful of typhoid culture produced a gallbladder infection. The impression is gained that for the successful experiments at least two loopfuls of culture were injected. In the light of these inconsistencies we feel that the conception of an arterial invasion of the gallbladder in rabbits inoculated with large doses of typhoid bacilli is neither proved nor disproved. Experiments and histologic studies to be reported in this paper make it certain that in addition to the common route, namely, the hemato-hepatogenous one, the transverse route via capillaries of the gallbladder wall may be responsible for the development of an experimental cholecystitis. Observations collected from nearly 500 necropsy examinations on rabbits, the results of which are recorded in paper IV in connection with the immunity experiments, and some cases of spontaneous rabbit paratyphoid have materially strengthened this conclusion. Moreover, it has already been proved by Rosenow,[67] that streptococci can invade the gallbladder wall in the form of emboli. The work of this writer leads, however, to a consideration of the last possibility by which bacteria can reach the biliary tract, namely, the lymphatics. From an experimental standpoint this route is of no importance. It is possible that the observations of Ledingham (see Morgan [50]), who noted typhoid bacilli in the gallbladder of guinea-pigs that had been injected intraperitoneally, can be explained on this basis. Clinically, it is an established fact that in the course of peritonitis or other abdominal infections (appendicitis) streptococci can be transported through the lymphatics to the biliary passages. The lymph vessels of the pancreas, periduodenal and peripyloric tissues, end in the fossa transversa of the liver; infection of the extra hepatic biliary system from these regions is therefore quite possible (Mix [75]). In a number of typhoid experiments we observed the frequent secondary infection of the gallbladder of rabbits with streptococci. In these cases the bile was sterile, while the wall gave on proper cultivation an abundant growth of indifferent streptococci. Coinciding with the observations of numerous surgeons (Baron [76]), we also noted in chronic cholecystitis of animals the displacement of B. typhosus by B. coli or by streptococci. A lymphatic invasion is the most likely route of these infections. The fact that the lymphatic spaces and vessels of the rabbit's gallbladder

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[73] Indian Jour. Med. Res., 1914-15, 2, pp. 1, 28 and 907.

[74] Ergebn. d. allg. Path. u. path. Anat., 1919, 19, pp. 351 and 471.

[75] Illinois Med. Jour., 1914, 25, p. 17.
[76] Beitr. z. klin. Chir., 1912, 77, p. 4[illegible].

or the extrahepatic ducts are seriously injured as a result of bacterial growth in the cystic bile or in consequence of mechanical injury to the biliary system lends support to this view. The so-called ascending route of infection is in all probability due to an invasion of bacteria through the lymphatics. The experiments of M. Müller,[77] with the typhoid bacillus in mice, moreover suggest that the spleen, liver, and their adnexa can readily become infected by way of the lymph stream. In animals with a native immunity against the micro-organisms this mode of invasion is the rule.

In summarizing these general considerations, it must be concluded that for the experimental production of a cholecystitis with intestinal organisms, such as the typhoid-dysentery bacilli and cholera vibrios, the hemato-hepatogenous route is the only one which has been definitely proved. An infection through embolic invasion of the wall may occasionally occur. This route demands, however, further experimental proof. An ascending infection follows, as a rule, the lymphatics; secondary or superimposed infections with streptococci take place through the same channels. The mechanism of bacterial transit from the interlobular veins or branches of the hepatic artery to the bile capillaries, and the factors which lead to a persistence of disappearance of the eliminated bacteria in the cystic bile and gallbladder, are as yet unknown. The facts presented later were collected with the desire to solve some of the questions which suggested themselves in the course of the critical analysis of our present knowledge concerning the problem of experimental typhoid cholecystitis in laboratory animals.

OPERATIVE PROCEDURES AND METHOD OF COLLECTING HEPATIC DUCT BILE FROM LABORATORY ANIMALS

For the experiments to be recorded in this and subsequent papers the sterility of the bile samples to be tested is of utmost importance; it is therefore self-explanatory that the operative procedures are conducted under strict asepsis. All animals used are completely etherized. To facilitate the exposure of the hepatic duct a thick pad is placed under the vertebral column between the costal and lumbar vertebrae. The shaved skin is thoroughly cleansed with soap and water, alcohol, ether, and painted with tincture of iodine. The body of the animal is always covered by sterile linen sheets and the field of operation is blocked by sterile towels, as customary for laparotomies.

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[77] Ztschr. f. Fleisch. u. Milchhyg., 1911-12, 22, p. 106; Centralbl. f. Bakteriol., I, 1912, 62, p. 335.

As a rule, a medium incision not longer than 5 cm. is made, extending from the xiphoid cartilage to the umbilicus. Occasionally in rabbits the viscera are exposed by a transverse incision extending along the rib margin of the right side. The latter method has, however, no advantage over the median incision. After the peritoneum is opened the left hand of the operator grasps with three fingers the pyloric region of the stomach and by bringing this portion gently into the opening of the abdomen, the hepatic duct is made visible. Particular care is necessary that during this act of the operation no mesenteric blood vessels are torn. The common duct is made accessible by fixing the small intestines with gauze pads soaked in warm saline. The mesentery of the duct is cut, avoiding the blood vessels, and a threaded French needle passed under the duct about 0.5 cm. from the duodenum. The tied thread enables the assistant to put the duct on the stretch. A second silk thread is then placed about 1 to 1.5 cm. from the first ligature; the duct is transversely incised with a small pair of sharp scissors and usually without any difficulty a glass cannula with a good neck can be inserted. The thread is then tied on the neck of the tube. When the cannula is properly placed, perfectly clear, slightly yellowish-green bile enters it as soon as the first ligature which holds the duct on a stretch is released. The gall cannula is connected by a fairly stiff rubber tubing, which is brought out either through the abdominal incision or through a stab wound on the right side of the abdominal wall. A series of silk sutures close the peritoneal cavity. The wound is covered with collodium.

The animal is then fixed on a padded and electrically warmed board. The rubber tube is connected with a sterile glass tube in a two-holed stopper, the latter being inserted in a pyrex test tube or an alkaline-free graduated centrifuge tube. The glass and rubber connections hold about 0.8 to 1.0 c c of bile.

The operation is easily performed on rabbits, and provided properly made cannulas are used. not only contamination but also admixture of blood is regularly avoided. Of about 80 rabbits operated on, with the technic and the aseptic precautions mentioned, one or two bile samples only revealed contaminating staphylo- or streptococci. In guinea-pigs, the technic of exposing the hepatic duct without hemorrhage is considerably more difficult, and blood-thinned bile specimens occurred in about 10% of the 30 animals operated on. As will be shown later, rats can be successfully prepared for hepatic bile collection, but the rate of flow is so slow that the animals operated on have to be kept in a fixed position for more than 12 hours. This prolonged period of collection produced in the two instances attempted, specimens contaminated by staphylococci.

The preparation of hepatic duct fistulas in dogs, cats, monkeys and goats offered no difficulties and in every instance sterile samples were obtained. It was, however, noted that the manipulations of the duodenum and of the liver caused regularly a prolonged period of reflex which inhibited bile secretion. With most of our dogs and cats we failed to obtain the necessary amount of liver bile without the use of cholagogues given either in form of ox bile by stomach tube 2 hours before the operation or more advantageously by injecting intravenously from 1 to 2 gm. of sodium taurocholate in warm saline solution. The cholagogue effect lasted usually from 2 to 3 hours; prolonged experimentation necessitated repeated injections. We are conscious of the fact that such procedures materially altered the constituents of the secretion, as will be shown later. The observations to be recorded on these biles should be viewed from this standpoint. The late Miss Foster of this laboratory has shown that about 90% of the injected taurocholic acid is eliminated in the fistular bile during the 2 to 4 hours following its injection. Numerous attempts to use bile obtained from dogs with simple gallbladder fistula were unsuccessful. The samples were regularly so badly contaminated with various micro-organisms that, in order to prepare suitable test specimens, repeated heating was necessary. The study of such samples naturally introduced new factors, which were not in the scope of our inquiry, and which dealt primarily with the bile freshly secreted from the liver.

In a number of rabbits the cystic duct was doubly ligated and cut before placing the cannula in the common duct. This operation is admittedly very delicate; utmost care is necessary to avoid injury or ligature of the cystic artery. The majority of our attempts resulted in hemorrhagic infarction, or minor circulatory disturbances in the veins of the gallbladder wall, which either became totally necrotic or showed hemorrhages and escape of blood into the lumen of the viscus. It is obvious that the injured tissues offer an excellent opportunity for localization of the intravenously inoculated typhoid bacilli. Our observations on the embolic invasion of the capillaries of the gallbladder mucosa were made on rabbits, which were neither laparotomized nor exposed to injury by ligation of the cystic duct or common duct.

The selection of the rabbits for the experiments to be reported followed the principles outlined in previous papers. If possible, members of the same litter were chosen. In about 1% of the rabbits and guinea-pigs employed spontaneous cholecystitis either due to B. coli (2) or streptococci (3) staphylococci (1) impaired the value of the experiment. The cystic bile was in these instances either distinctly changed in color or sections of the thickened wall revealed a diffuse lymphocytic infiltration of the mucosa and submucosa. Occasionally the bile appeared to be normal, while the histologic picture exhibited a low grade infection of the lymphatics of the mucosa and subserosa. The preparation of the suspension of typhoid bacilli to be injected, the cultivation of the bile and blood specimens and the numerical determination of the bacteria in the tissues were treated in the same manner as stated in previous papers.

EXPERIMENTAL DATA

From the discussion of the present knowledge concerning the possible path by which the typhoid bacilli on intravenous inoculation reach the gallbladder, it is evident that the hemato-hepatogenous system is probably the usual route. The factors which favor the transit of the bacteria from the blood to the bile are, however, incompletely investigated. Moreover, nothing is known relative to the exact number of typhoid bacilli, which enter the biliary system in this manner, and their fate in the biliary secretion. The experiments thus far published (with the exception of those by Nichols) deal with the phase of the problem only in a qualitative manner. We are by no means convinced that the mere presence of bacteria in the bile, after an intravenous injection of typhoid bacilli in a sufficiently large number to ensure passage into the excretion, leads always to a cholecystitis or even to a temporary persistence of the organisms somewhat analogous to the human carrier state. Furthermore, one should know how soon after the injection and for what period of time the bacilli are discharged in the bile. In the preceding paper the fate of the typhoid bacilli was determined by cultivating the tissue from 1 to 160 hours after the injection. No attention was paid at that time to the behavior of the bacilli in the blood stream during the first hour. Bull [78] and others have already considered this phase of the problem, but in connection with the study of the disappearance of the leukocytes from the peripheral blood stream of animals with biliary fistulas that had been intravenously inoculated with living typhoid bacilli, the fate of these organisms in the blood vessels was simultaneously investigated.

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[78] Jour. Exper. Med., 1914, 20, p. 237; 1915, 22, pp. 475 and 487; 1916, 23, p. 419; 24, p. 25.

The work of Nichols suggested a relationship between immunization and elimination of the injected bacteria. In the experimental study of this phase the results of this writer have in part been confirmed and amplified by our work on guinea-pigs.

THE ELIMINATION OF TYPHOID BACILLI IN THE HEPATIC DUCT BILE OF NORMAL AND IMMUNIZED RABBITS, GUINEA-PIGS AND DOGS

Charts 1 and 2 illustrate the rate of elimination of typhoid bacilli in the hepatic duct bile collected periodically from common duct fistulas of 2 normal and 2 immunized rabbits. The degree of leukopenia and the rate of disappearance of the inoculated bacilli are also demonstrated. The observations have been collected from a series of 8 successful experiments on coccidiosis-free animals. The cystic duct has always been tied before placing the hepatic duct cannula, and the animals have been permitted to recover from the operation (at least 2 to 3 hours) before the bacteria have been inoculated. Varying amounts of cultures grown on agar have been introduced intravenously. When the number of bacteria injected has been less than 1,000 million, the plates prepared from the bile specimen collected for a period of 2 hours have never shown more than 10 bacteria (table 1).

TABLE 1

ELIMINATION OF TYPHOID BACILLI IN HEPATIC DUCT BILE IN IMMUNIZED AND NORMAL RABBITS

Experiment	Rabbit	Number of Typhoid Bacilli Inoculated	Total Colonies in Bile	Time of Collection
1	1114a Immunized.................	600.000,000	6 in 21.9 c c	120 Min.
	1114b Normal......................	600,000,000	7 in 24.1 c c	120 Min.
2	1147a Immunized.................	10.000 million	12 in 20.35 c c	120 Min.
	1147b Normal......................	10,000 million	28 in 25.9 c c	120 Min.
3	1141a Immunized.................	12,000 million	13 in 15.3 c c	60 Min.
	1141b Normal......................	12,000 million	183 in 15.6 c c	60 Min.
4	1148a Immunized.................	20,000 million	23 in 7.4 c c	120 Min.
	1148b Normal......................	20,000 million	422 in 10.7 c c	120 Min.
5	1136a Immunized, 1½ year old..	24.000 million	3,377 in 36.6 c c	120 Min.
	1136b Normal, 1½ year old.......	24,000 million	24.609 in 27.0 c c	120 Min.

Inoculations of large doses varying from 10,000 to 24,000 million produce a rapid discharge of typhoid bacilli. In one instance it was profuse. With the exception of exper. 2, the general character of the curve of elimination has followed the one given in chart 1. In the normal animal the bacilli appear in greater numbers than in the immune. There are some differences in elimination characteristics for certain

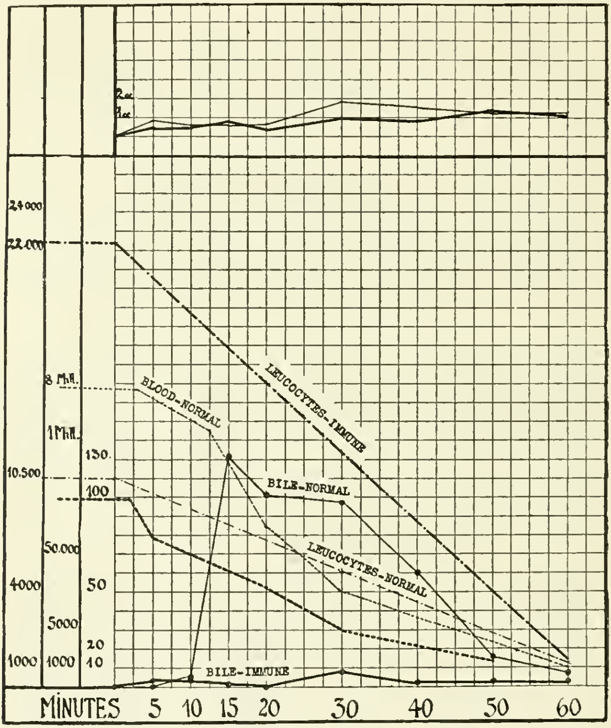

Chart 1.—Rate of elimination of typhoid bacilli in hepatic duct bile in 1 normal and 1 immunized rabbit. Degree of leukopenia and disappearance of bacilli from bloodstream. Rate of bile flow during the experiment.

litters, depending on the age of the individual animals. The age of the culture, whether grown with or without rabbit blood, has no influence on the total number of bacilli discharged in the bile. In every instance but one the cystic bile has been sterile.

As a rule, the elimination of the bacilli is immediate. Making an allowance for the bile which is present in the cannula at the time of the intravenous injection, it is obvious that less than 5 minutes elapse before the bacteria reach the biliary passages. Moreover, the maximum discharge occurs in the first 5 to 15 minutes, in some instances even in less time. In subsequent periods the number decreases rapidly and not infrequently ceases completely at the end of 1 hour. While these observations confirm the facts already reported by v. Fütterer, Biedl and Kraus, Blumenthal and Nichols, in very exceptional cases, as has been exemplified in the data published by Doerr, and by ourselves (paper IV), the elimination is delayed or even on repeated inoculation is not demonstrable. According to one of Doerr's tables (footnote 1, page 629), at least 8 hours elapsed before the typhoid bacilli inoculated in an amount of 2 standard loopfuls, an average of 5,000 million organisms, were present in the cystic bile of rabbits. Three of his animals in the same series killed on the 2nd, 4th, and 6th hours gave sterile cystic bile specimens. Doerr has failed to state whether the secretions have only been plated or whether they have also been enriched. On several occasions it has been noted that even repeated inoculations of large doses of typhoid bacilli have resulted either in sterile hepatic duct bile specimens, or the number of organisms demonstrated by plating has been below 10 and has been insignificant in proportion to the inoculum. This statement is best illustrated by the presentation of an experiment.

Exper. 9.—Normal rabbit 1059 (weight 2,525 gm.) was inoculated with 7,750,000,000 typhoid bacilli (polyhomogenous 5 strains) after his cystic duct had been ligated and a common duct fistula placed. During the initial period of 60 minutes no bacilli were discharged. At the end of the first hour an additional inoculation of 16,000,000,000 bacteria was made and the collection of the samples continued for 60 minutes. A total of 7 colonies was counted in specimens obtained 100, 110 and 120 minutes after the first injection. The collecting tube contained 2 organisms, while 0.8 c c of deep green, blood-free cystic bile gave 396 colonies. The gallbladder wall registered 23,000 typhoid colonies per 100 mg. of tissue.

The leukocytes dropped from 18,000 to 1,300 per c c, and the blood freed itself of the bacteria as follows: 1 minute after the 1st injection, 1,400,000 per c c; 60 minutes after the injection, 30,000 per c c; 1 minute after the 2nd injection, 19,000,000 per c c; 60 minutes after the injection, 660,000 per c c in the peripheral blood and 100 c c in the heart blood.

It is evident from this and similar experiments that (1) even a large dose of typhoid bacilli in a limited number of rabbits never leads to a discharge of the bacilli by way of the hemato-hepatogenous route; (2) the gallbladder bile may become infected even after ligation of the cystic duct; and (3) particularly if one considers the tremendous dosage and the rapidity with which the blood stream freed itself of organisms, the transit of typhoid bacilli in the liver from the blood to the bile capillaries in the first 2 hours after the injection cannot be the outcome of a mechanical rupture of the capillary walls by the masses of bacteria resulting in a flushing of the organisms into the biliary secretion.

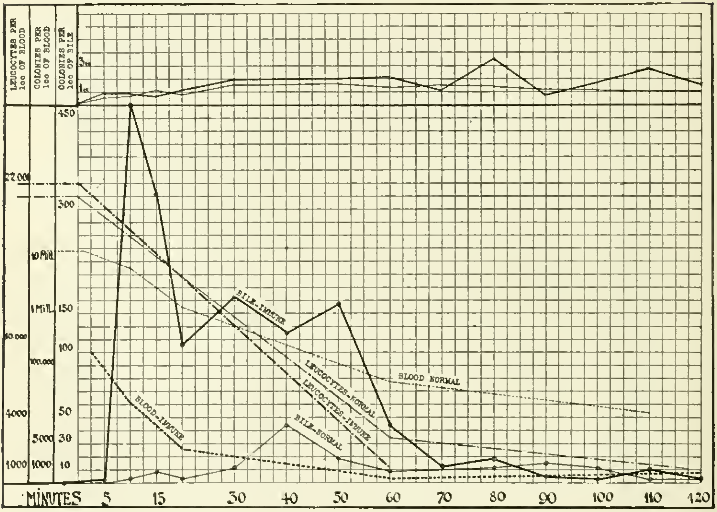

Chart 2.—Elimination of typhoid bacilli in the duct bile of a normal and of a recently immunized rabbit.

Additional experiments confirm the foregoing conclusions as far as they concern the infection of the gallbladder whose cystic duct has previously been ligated. Inoculations of large doses of typhoid bacilli may cause an infection of the cystic bile in animals with a ligated cystic duct. Our experiments were originally conducted according to the procedure of Doerr. Nine rabbits were operated on; the cystic duct was carefully tied; and the animals permitted to recover from the laparotomy. After a lapse of from 4 to 6 days, they were inoculated with a sublethal dose of B. typhosus (varying from *2* to 4,000 million). Twenty-four to *72* hours after the injection, the animals were killed and their tissues cultivated. Five animals must be eliminated from consideration on account of the profound changes in the gallbladder wall (hemorrhages, partial necrosis, etc.). Three of these rabbits had sterile cystic biles while the remaining *2* showed varying numbers of typhoid bacilli. Four rabbits were successfully operated on; the bile at necropsy was deep olive green and viscid; the gallbladder wall was microscopically intact. Neither of these animals gave a positive cystic bile culture and the gallbladder wall was either sterile or contained only a few bacilli. Moreover, the duodenal content and mucosa of these rabbits was sterile or failed to give specific colonies. On first consideration these experiments seem to confirm the experiences of Doerr

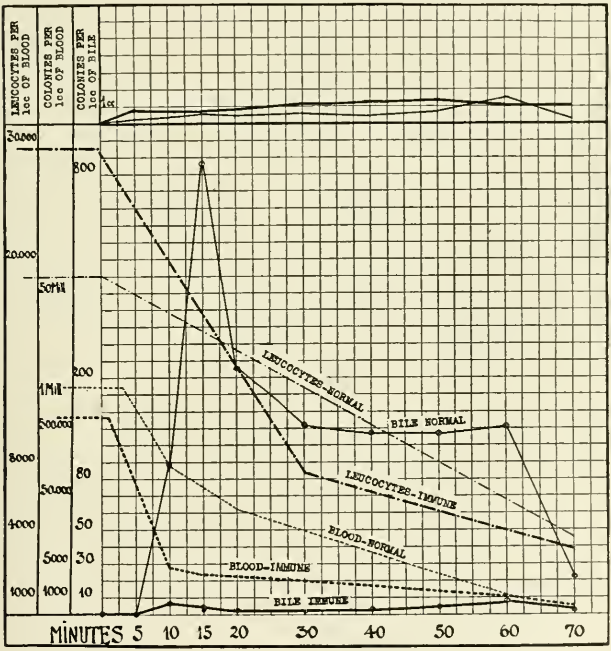

Chart 3.—Elimination of typhoid bacilli in the duct bile of a normal and of an immunized guinea-pig.

and Blumenthal, but on further comparison with other data at our disposal considerable hesitancy is felt in drawing far-reaching conclusions. The absence of typhoid bacilli in the duodenum deserves in this connection some consideration. Laparotomized rabbits succumb readily to a typhoid intoxication in the first 12 to 24 hours, as has been stated repeatedly. It is technically impossible to infect such animals with recently isolated typhoid strains in amounts which ensure elimination in the hepatic duct bile and which lead to an invasion of the gallbladder wall. In numerous experiments on rabbits which were not previously operated on it was noted that only 40% of the animals developed infected gallbladders, when injected with the same technic as in the above tests. The experimental data recorded in animals previously laparotomized are therefore seriously invalidated and cannot be used in any argument against the theory of embolic wall infection. Exper. 9, previously described, suggests a means of overcoming these technical difficulties. In these experiments a number of rabbits were laparotomized, the cystic duct carefully dissected, ligated and cut, and a common duct cannula inserted. After a lapse of from 3 to 5 hours, they were injected intravenously with large amounts of typhoid bacilli (15 to 24 billion, one slant of peptic digest of ½ slant of rabbit-blood agar cultures). Bile specimens were collected for from 2 to 8 hours, and at the end of this period the animals were killed. Careful cultures revealed in 2 of the 6 rabbits successfully operated on, 69 and 117 typhoid colonies in 0.4 and 0.7 c c, respectively, of deep green bile. Neither of the 2 specimens contained traces of blood; the gallbladder walls gave 8,700 and 6,900 colonies, respectively, per 100 mg. of tissue. During the observation period of 8 hours, 3,300 and 1,280 typhoid bacilli were eliminated by the liver. These experiments suggest that in a number of rabbits typhoid bacilli can reach the cystic bile by way of the blood vessels of the gallbladder wall provided a sufficiently large inoculation is given. The manner in which the bacilli enter the bile will be discussed in connection with the consideration of the microscopic findings in these gallbladders.

It is definitely shown in chart 1 and table I that the immunized rabbit discharges less organisms than the normal animal. This fact is even more strikingly shown in chart 3 illustrating the same type of experiment in a normal and immunized guinea-pig. The graph has been prepared from the data of an experiment selected from a series of 4. The study conclusively proves that in the immunized guinea-pig either no bacteria or few pass into the biliary capillaries. The result depends entirely on the number of bacilli inoculated, as is shown by the following figures:

TABLE 2

NUMBER OF BACTERIA IN HEPATIC DUCT BILE OF IMMUNIZED AND NORMAL GUINEA-PIGS AFTER INOCULATION OF TYPHOID BACILLI

B. Typhosus Injected	Time after Injection	Immunized	Normal
100,000,000	120 Minutes	0	2,216
300,000,000	60 Minutes	11	1,942
450,000,000	60 Minutes	33	239
600,000,000	60 Minutes	45	1,680

As the immunized guinea-pigs can destroy typhoid bacilli more readily than the normal animal, a difference which is not demonstrable in the rabbit, it is not at all surprising to find the contrast we have noted in the elimination of bacteria. Immunization obviously produces in the blood vessels of the liver some factors controlling the elimination of the organisms which can only be overcome by a large inoculation. The nature of these factors is not definitely established, but we have been able to demonstrate in the guinea-pig histologically a very active phagocytic action of the endothelial cells. At the end of 2 hours most of the Kupffer cells are packed with typhoid bacilli. The same phenomenon occurs also in the rabbit but to a less marked degree. The endothelial lining of the liver capillaries, which is endowed with the maximum ability to phagocytize and to destroy typhoid bacilli, will also be resistant to the typhoid toxins, which tend to injure the capillary wall and permit in this manner an escape of some bacteria into the adjacent biliary capillaries. The guinea-pig is relatively insusceptible to the typhoid toxin[79] present in the bacterial emulsion. The maximum elimination of the bacteria in the hepatic duct bile occurs in the first 10 to 25 minutes after the injection, corresponding to the time when the liver has accumulated approximately from 25 to 40% of the bacterial masses inoculated. In this connection one recalls the observations of Helly[80] and of Schwarz,[81] who have found typhoid bacilli not only inside, but outside of the capillaries in the pulp cords and the malpighian bodies of the spleens of guinea-pigs 10 minutes after an intravenous injection. The capillary system in general, and not only that of the liver vessels, is therefore permeable to bacteria shortly after a drastic intravenous injection. In the immunized guinea-pigs polymorphonuclear and endothelial phagocytosis begins immediately, and comparatively few organisms can be found free in the blood stream. In accordance with this fact, the number of typhoid bacilli per c c of peripheral blood of the immunized guinea-pig is less in the first 20 minutes than in the normal animal. As long as the bacterial masses to be phagocytized are comparatively small and can be handled by the cells destined for this purpose, no bacteria will enter the bile. In case the injection and the subsequent hepatic invasion are overwhelming, a transit of organisms will be recorded. The elimination is apparently not an excretory process. This statement is based on the following observation. The foregoing experiments were repeated after adding to the bacterial suspension a 1% Congo-red solution in saline (1 c c in guinea-pigs and 10 c c in rabbits, Cecil and Weil[82]). The dye appeared in the normal and immunized animal in from 4 to 18 minutes. No differences in the amounts of the stain excreted could be determined in the 2 animals, but numerous typhoid bacilli were present in the bile collected from the normal, while sterile plates were obtained from the immunized guinea-pigs. In some instances the excretion of the dye preceded the appearance of bacteria in the bile by from 8 to 15 minutes. The results just mentioned suggest that a further study of the transit of bacteria from the blood to the bile capillaries be combined with an analysis of the excretory function of the liver of the same animal by some similar test. All these observations constitute a strong argument in support of the conception of a cellular immunity, particularly when tests are conducted on guinea-pigs which have been vaccinated several months previous to the tests and which have lost their serum immune bodies. The number of such experiments is relatively small, but definitely indicates that a guinea-pig once treated with typhoid bacilli will always behave like an immunized animal even when the usual tests suggest that his immunity has been lost. A persistent experimental typhoid cholecystitis can only be produced in immunized guinea-pigs with very heavy intravenous infections.

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[79] For a detailed consideration of the so-called typhoid toxin consult the recent summary of H. Zinsser, Jour. Immunol., 1902, 5, p. 265.

[80] Beitr. z. path. Anat. u. z. allg. Path., 1903, 34, p. 387.

[81] Ztschr. f. Heilk., 1905, 26, p. 295.

In the rabbit the endothelial cells of the liver capillaries function less vigorously than in the guinea-pig, but differences in activity of the cells of the normal and of the immunized animals can be detected by the bile elimination test, provided a sufficient time has elapsed between the last immunizing dose and the test injection. Immunized rabbits injected with small doses of typhoid bacilli on the 6th (chart 2) or 10th day (Nichols, footnote 27, 504) discharge more bacteria than normal ones. The endothelial barrier is apparently imperfect; the cells have in consequence of the vaccination not sufficiently regenerated or acquired the function of "immunity," and bacteria leak in to the bile capillaries. The observation has been confirmed repeatedly. Irrespective of the agglutinin content of the blood or the number of injections, the bile of immunized rabbits infected before the 6th to 10th day contains more bacilli than the same secretion of normal rabbits.

[82] Jour. Am. Med. Assn., 1917, 69, p. 521.

For example, rabbit 1374 was immunized intravenously by 10 inoculations of heat-killed typhoid bacilli. Six days after the last injection, when the agglutination titer was 1:200,000, the animal, together with rabbit 1090 (a "recovered carrier" with an agglutination titer of 1:200) was inoculated with 8.5 billion living typhoid bacilli. Killed after a lapse of 2 hours, the bile of rabbit 1374 contained 800,000 typhoid bacilli per 3.3 c c, while 1.2 c c bile of rabbit 1090 proved sterile on direct plating and 0.4 c c of the same on enrichment in broth was likewise sterile. The destruction of the bacteria in the tissues of both animals was practically identical. The blood contained 200 and 300 typhoid bacilli per 1 c c of blood.

Our experiments fully explain one observation made by Nichols. One of us (K. F. M.) immunized the rabbit used by this writer. According to the available records, the test injection was given on the 5th day after the last immunizing inoculation. The conclusion of Nichols, that more bacilli appear in the bile after an injection of the same dose in immunized animals than in normal animals, deserves some modification. It should read "immunized animals which have not recovered from the treatment discharge more bacilli." The facts elucidated by the experiments may have some bearing on the observations of Metchnikoff and Besredka,[83] that anthropoid apes are not protected against an alimentary typhoid infection when the animals are tested in from 4 to 6 days after finishing the immunizing treatment. They partially explain our own experiments reported in paper III: namely, immunized rabbits infected on the 10th day after the injection of the last dose of vaccine develop in a higher percentage of instances gallbladder infections than nonprotected animals. It is not unlikely that the reports of Creig[84] and Flu[62] on rabbits developing gallbladder infections in the course of immunization with cholera-like vibrios and Flexner dysentery bacilli may in part be explained by the foregoing fiindings. The question naturally arises, what constitutes a safe period for recovery? Until recently, all processes of immunity have been gaged by the appearance or disappearance of immune bodies in the blood serum, while little or no attention has been paid to the reparative changes which by necessity must take place in the injured tissues. Nobody will deny that heat-killed typhoid vaccines produce, for example, liver necrosis, proliferation of macrophages, etc., but how many days are necessary to cause a restitutio ad integrum fails to interest the serologist, who studies disease and infection only by test-tube experiments. It is a matter of common knowledge in our laboratory that a rapid method of immunization, for example, the procedure suggested by Fornet and Müller, by Bull[85] and by others, produces immune serums of great potency with no loss in animals and no gallbladder infections. On the other hand, the "standard method" of immunization, at 7 to 10 days, in comparison furnished poor serums, frequent losses of animals from cachexia and numerous gallbladder infections. The rapid method of immunization of rabbits finds, therefore, some advantageous justification in view of these data.

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[83] Ann. d. l'Inst. Pasteur, 1911, 25, p. 193

[84] Indian Jour. Med. Res., 1915-16, 3, pp. 259 and 397.

The elimination of typhoid bacilli in the hepatic duct bile of dogs has been studied in 10 animals. The results have been inconstant. Differences between the rate and degree of bacterial elimination in immunized and normal dogs have been indefinite. Injections of less than 8,000 million have failed to cause transit of bacteria, in dogs varying in weight from 14 to 26 pounds. Cats behave in a similar manner. It is generally known from the studies of Cushing[86] and our own, that typhoid bacilli introduced directly into the cystic bile of dogs disappear rapidly and that a cholecystitis can only be produced by considerable injury of the wall or by placing a foreign body in the gallbladder (Marxer[16]). A more careful study of the mechanism of gallbladder infection in these species of animals has therefore been considered superfluous.

THE DISAPPEARANCE OF THE TYPHOID BACILLI AND OF THE LEUKOCYTES FROM THE PERIPHERAL BLOOD STREAM

Blood samples were collected from the ear veins at regular intervals during the experiments on fistula animals. By means of pipettes with a capacity of 0.01 c c the blood drops collecting on a sterilized area of the ear were aspirated. Leukocyte counts were obtained from the same specimens. For each test a fresh incision was made. Hypocoagulability favored the collection of blood and as long as the blood pressure was normal, uncontaminated cultures were obtained. The findings are included in charts 1, 2 and 3.

In a general way, the observations confirm the well-known fact, originally established by Wyssokowitch[72] and von Fodor,[87] that bacteria intravenously inoculated disappear rapidly from the circulation. The analysis of the data presented in paper IV indicates that at least one hour is necessary to cause a complete absence of the typhoid bacilli from the heart blood, obtained from living animals by puncture. Furthermore, it has been shown that the organisms are deposited in the liver, spleen, bone-marrow and lung. At the end of from 1 to 2 hours the bacteria may reappear and circulate in moderate numbers until the 4th to 8th day. In paratyphoid infected animals the period is even longer and in case the animals succumb the bacilli may be demonstrated in the blood in large numbers at the time of death. The disappearance of the typhoid bacilli is decidedly more rapid in the immunized animals, but not as complete as demonstrated by Bull. Our results differ from those reported by this writer for these reasons: (a) a heavier dose of bacteria was injected; and (b) the blood samples were collected from the peripheral vessels instead of from the heart.

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[85] Jour. Exper. Med., 1916, 24, p. 25.

[86] Bull. Johns Hopkins Hosp., 1899, 10, p. 166.

[87] Arch. f. Hyg., 1886, 4, p. 129; Von Fodor, I., and Rigler, F : Centralbl. f. Bakteriol., I, 1898, 23, p. 930.

The mechanism of the removal of bacteria which is the same even on repeated injections (exper. 9) has been the subject of considerable study. It is interpreted by Bull[88] to be a result of in vivo agglutination, but recently Debres and Govaerts[89] attribute the clumping of the intravascular bacteria to the action of the blood platelets. The intravenous inoculation in their opinion causes a disturbance of the plasma with an agglomeration of platelets, which in turn engulf the bacteria. The agglomerated masses vanish from the blood stream to be deposited subsequently in the capillaries, sinusoids, etc., of the organs. The removal of the blood platelets produces in turn a hypocoagulability. We have had occasion repeatedly to note the prolongation of the coagulation time of the blood collected 15 to 20 minutes after the inoculation of living or dead typhoid bacilli. The following facts also speak against an in vivo agglutination. According to Ten Broeck[90] and our own observations, virulent and invasive hog cholera or paratyphoid bacilli are rapidly clumped and disappear from the blood even though the blood contains no demonstrable agglutinins, while Hopkins and Parker[91] noted rapid removal of virulent hemolytic streptococci without any evidence of agglutination. In vivo agglutination may be positive even when in vitro experiments fail to demonstrate this property. It is the recognition of these exceptions which casts doubt on the correctness of Bull's interpretation of the mechanism explaining the phenomenon of removal of bacteria from the blood stream.

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[88] Jour. Exper. Med., 1915, 22, pp. 475 and 487.

[89] Comp. rend. Soc. de biol., 1918, 81, p. 53; Presse méd., 1918, 26, p. 597

[90] Jour. Exper. Med., 1917, 26, p. 441

[91] Ibid., 1918, 27, p. 1.

In normal guinea-pigs the bacilli are loosely aggregated without being truly agglutinated and yet they promptly collect in the lung, liver, spleen, etc. In a general manner one gains the impression from properly stained smears prepared from the organs that a true agglutination has not taken place, but that physical disturbances in the colloidal suspensions, the blood and the bacteria, are responsible for the aggregations of bacilli. In some respects it is difficult to distinguish agglutinations in vivo from the phenomenon of the concentration of foreign particles in the viscera observed by Werigo,[92] in normal animals. In immunized rabbits and guinea-pigs the removal of bacteria takes place more rapidly, but we fail to note any differences in the formation of the clumps, which would definitely indicate true agglutination. As a rule, the aggregations are not more compact, nor do they consist of more bacteria. Our observations record the fact that the bacteria disappear from the circulation and that this disappearance is frequently followed in the normal animal by the discharge of the organisms into the hepatic duct bile. Whether this removal is the result of in vivo agglutination or the action of the blood platelets, or purely a dispersion phenomenon of the two colloids, has to be decided by further investigation.

The behavior of the leukocytes as illustrated in charts 1, 2 and 3, deserves some consideration, as it may assist in the explanation of the ultimate fate of the bacilli accumulated in the viscera. The observations made on animals which, on account of the hepatic duct fistula, exhibited a postoperative leukocytosis, confirm the well-known fact that the intravenous injection of bacteria or foreign protein causes a leukopenia in the peripheral circulation notwithstanding the postoperative leukocytosis. This condition is again followed in a few hours by a considerable leukocytosis. In the smears 10 to 15 minutes after the injection the majority of the white cells were mononuclear forms. The studies of Goldscheider and Jacobs,[93] Werigo,[92] Schwarz,[81] C. W. Wells,[94] Sellards and Baetjer,[95] Jolly,[96] Nagao [97] and others lead to the conclusion that the leukopenia is the result of the accumulation of the polymorphonuclear leukocytes in the internal circulation, especially in the liver, spleen and lungs. The view of an uneven distribution of the leukocytes in experimental typhoid infections is, however, not shared by Studer,[98] by Tachigara and Miura,[99] by Pepper and Miller,[100] and by others, mainly on account of their inability to demonstrate at the low point of the leukopenia an accumulation of leukocytes in the internal organs. The leukopenia is, according to Studer, who injected his animals subcutaneously, the result of a functional disturbance provoked by the typhoid toxin on the bone-marrow and on the lymphatic tissues. A similar opinion has been expressed by Tachigara and Miura. In this connection we recall some observations made in the course of studies on hyperleukocytosis (Hurwitz and Meyer,[101]) following the injection of living typhoid bacilli. During the period of leukocytosis the increase in the percentage of young forms of leukocytes or metamyelocytes is so great as to suggest that during the leukopenia some of the leukocytes are destroyed or that the typhoid bacillus injures the leukopoietic tissue, namely, the bone-marrow. This peculiar blood picture is frequently more marked in animals which have been injected with living typhoid bacilli. There are several explanations of the purpose of the localization of leukocytes in the viscera. The theory of a negative or repellant chemotaxis according to which the introduction into the general circulation of a foreign protein repels the polymorphonuclear leukocytes, causing them to find refuge within the capillaries of the internal organs, is theoretically possible. There is some doubt in the opinion of numerous observers as to the actual occurrence of a negative chemotactic action on leukocytes. The evidence adduced from our findings supports the conception of a positive chemotaxis in the viscera as advanced by C. W. Wells, Nagao (footnote 97, p. 351), and others. The disappearance of the polymorphonuclear leukocytes from the blood stream was frequently preceded by the clumping of the bacteria and their removal to the liver, spleen, etc. This fact is best shown in table 3.

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[92] Ann. de l'Inst. Pasteur, 1892, 6, p. 478.

[93] Ztschr. f. klin. Med., 1894, 25, p. 373.

[94] Jour. Infect. Dis., 1917, 20, p. 219; 1918, 20, p. 502.

[95] Bull. Johns Hopkins Hosp., 1918, 29, p. 135.

[96] Compt. rend. Soc. de biol., 1918, 81, p. 756.

[97] Jour. Infect. Dis., 1920, 27, p. 327.

Smears from the lung and liver demonstrate the well-known phagocytosis described by Bull.[84] It is obvious that the leukocytes perform a definite function, at least during the first 2 hours after an intravenous infection, in removing and distributing to the detoxicating organ—the liver—a considerable proportion of the typhoid bacilli. The bacterial protein liberated during the course of phagocytosis and phagolysis may exert an additional chemotactic effect on the leukocytes or may in turn injure the bone-marrow, as suggested by Studer and others. The latter point can, however, not be decided from the data at our disposal, and the action does not occur during the first 2 hours.

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[98] Thesis, University of Zürich, 1903.

[99] Mitt. a. d. med. Fakult. a. k. Univers. z. Tokyo, 1917, 17, p. 539.

[100] Jour. Infect. Dis., 1916, 19, p. 694.

[101] Jour. Exper. Med., 1916, 24, p. 515.

This interpretation of the leukocyte counts affords no support for the conclusion that the leukopenia in typhoid fever is explained on the same basis. As far as the leukocyte counts of the peripheral blood is concerned, the available evidence indicates that the absolute diminution is much less than one has ordinarily supposed. Moreover, the spleen tumor is not the result of an accumulation of leukocytes as assumed by C. W. Wells, but due to a hyperplasia and activity of the reticulo-endothelial macrophages accompanied by a toxic inhibition of the leukopoietic function as stated by F. A. Evans,[102] S. Gräff [103] and others. Moreover, the recent observations of Askanazy [104] and of Gräff indicate that the typhoid bacillus and its toxin exert primarily specific positive chemotactic influence on the endothelial cells, while the leukocytes only become engaged later in the course of the disease when the necrotic tissues saturated with bacterial protein act as chemotactic foreign bodies. This local accumulation of leukocytes for the purpose of digestion of the necrobiotic foci has no appreciable influence on the blood picture of the peripheral blood.

TABLE 3

RABBIT 1265 INJECTED WITH 345,000,000 TYPHOID BACILLI. 1 C C OF THE SERUM OF THIS RABBIT DESTROYS IN 5 HOURS 1,000,000 TYPHOID BACILLI

Time After Injection	Heart Blood Contained	
	Bacteria per C c	White Cells
30 seconds	3,200,000	11,300
2 minutes	800,000	14,600
4 minutes	400,000	10,500
10 minutes	34,000	
11 minutes	34,800	4,200
15 minutes	6,500	2,600
20 minutes	800	1,400

Experiments on rabbits even when conducted with paratyphoid bacilli cannot be chosen for a reproduction of the condition in man. The removal of the bacteria and the subsequent leukopenia resulting from an intravenous injection is nonspecific and can be observed following the injection of any micro-organism. Even the progressively invasive paratyphoid bacilli are actively phagocytized during the period of leukopenia, when the leukocytes and the clumped bacteria are brought in intimate contact. The ultimate fate of the gram-negative bacilli in the viscera may depend on some mechanism other than the ingestion by polymorphonuclears, namely, the endothelial cells or the bactericidal substances liberated by the leukocytes may play an important rôle in this problem.

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[102] Bull. Johns Hopkins Hosp., 1916, 27, p. 356.

[103] Deutsch. Arch. f. klin. Med., 1918, 125, p. 352; and 126, p. 1.

[104] Askanazy, M.: Deutsch. med. Wchnschr., 1916, 42, p. 897.

CHOLECYSTITIS AS A RESULT OF GALLBLADDER WALL INFECTION

In the course of nearly 500 necropsies on rabbits infected by intravenous injection of typhoid bacilli, we noted in about 25% of the animals extensive inflammatory processes in the gallbladder wall. This organ was frequently adherent to the abdominal wall, the liver lobes, the stomach or even to the large intestines. Some rabbits killed from 8 to 10 days after the injection exhibited either a diphtheritic, necrotized or perforating gallbladder wall, the lesions being prominent particularly in the fundus region. Numerous small mural abscesses associated with hemorrhages were occasionally evident. In some experimental series the lesions named in the foregoing were more frequent than in others; in fact, in one series of 48 rabbits, the inoculation of agglutinated living typhoid bacilli produced in the gallbladder of 5% of the animals, blood-tinged biles with small or large blood clots. In a number of instances hemorrhagic ulcers or erosions were noted in the jejunum of the same animals. Histologically, the lesions originated from the terminal blood vessels of the mucosa. These and similar observations, the experimental results reported in paper IV, and the findings collected from rabbits infected intravenously with paratyphoid B. bacilli (Litch and Meyer [43]) left no doubt that the gallbladder wall is often seriously damaged in the course of a typhoid or paratyphoid infection. Careful culture studies supplied, moreover, data which indicated that gallbladder biles might be found sterile, while the walls yielded innumerable specific colonies. Furthermore, the findings of a large percentage of cholecystitis cases among immunized rabbits did not harmonize with our conception of a hemato-hepatogenous route of infection. It was obvious that the small number of typhoid bacilli which was eliminated in the bile could pass directly through the hepatic duct into the intestines and might never reach the gallbladder. These and other considerations made it apparent that the conception of a hemato-hepatogenous route of biliary infection did not explain all of our results. An attempt was therefore made to verify the studies of Chiarolanza,[48] who demonstrated nests of bacilli lying in relation to the capillaries of the gallbladder wall, from which source the bile was apparently invaded.

The experiments of J. Koch and Chirolanza [48] have already been discussed and it has been concluded that for technical reasons they are open to serious criticisms, as are those dealing with the ligated cystic duct reported by R. Doerr.[1] A histologic study of the gallbladders removed at various periods after the infection has therefore been chosen as the most promising method of determining the possibility of infection of the bile by way of the gallbladder wall. Such an analysis gives not only definite information relative to the theory of wall infection, but also furnishes facts which help in the explanation of the exclusive persistence of typhoid bacilli in the gallbladder.

The literature contains reports of the histologic studies of Weinfurter,[58] Hailer and Ungermann,[105] Emmerich and Wagner,[106] Violle [15] for typhoid, and those of Creig [73] for cholera cholecystitis of the rabbit. As a rule, the chronic stage of the infection has been studied. The majority of writers mention cellular infiltration with collections of bacteria in the mucosa, and accept unreservedly the descriptions and interpretations of Chiarolanza. An excellent description of the gallbladder lesions found in rabbits infected with a specific cholecystotropic paratyphoid bacillus has been published by Fränkel and Much.[69] Bacterial thrombi have been observed by these investigators in the capillaries of the mucous membrane either at the top or at the base of the folds. Frequently wall abscesses and bacterial masses have been noted in the lymph vessels. These reports are in marked contrast to the repeated statements of Nichols [107] that he has been unable to find bacterial foci in the gallbladder wall.

In the course of our infection experiments we systematically collected and fixed the gallbladders, while warm, in Zenker's solution. Sections were stained with hematoxylin-eosine, or with dilute Manson's blue or carbolthionine. The gallbladders of 132 rabbits have been examined, but a few characteristic sections are chosen for a discussion of the most important findings.

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[105] Arb. a. d. k. Gsndbtsamte, 1914, 47, pp. 303, 451 and 470.

[106] Centralbl. f. allg. Path. u. path. Anat., 1916, 27, p. 433.

[107] Jour. Am. Med. Assn., 1917, 68, p. 958; Nichols, H. J.; Simmons, I. S., and Stimmel, C. O.: Ibid., 1919, 73, p. 680.

The description of the microscopic structure of the normal gallbladder, as reported by Violle [15] and others, deserves one addition. In the majority of the gallbladders we found the villi of the mucosa located in the fundus portion to be long and irregular (fig. 1), resembling a papillomatous growth. The mucosa of the neck and the lateral walls, in contrast, have short and stumpy villi (fig. 2). The epithelium always exhibits a cuticular border, the cells are frequently of the goblet type, and in properly fixed material the lining is perfectly intact.

The pathologic changes, which we found in looking over many hundreds of sections, are definite and conclusive. In this connection it should be emphasized that the lesions to be described can only be found in 25% of the rabbits inoculated into an ear vein or by way of a radical of the portal vein. This statement applies particularly to the important lesions which develop between the 6th and the 72nd hour. The course of the wall infection can be discussed after studying the photomicrographs (in figs. 1 to 12), and is briefly as follows:

At the end of the 6th hour, after an intravenous injection of at least 5,000 million organisms in a rabbit weighing 2500 gm., the gallbladder bile may be sterile, but the wall may contain thousands of typhoid bacilli. A scrutiny of the villi of such a gallbladder may reveal dilated capillaries, perhaps nests of leukocytes and an edematous infiltration of the perivascular tissue (fig. 2). Typhoid bacilli can occasionally be demonstrated in the vascular endothelium, but quite regularly in the Kupffer cells of the liver. At the end of 24 hours a hemorrhage may occupy the tip of the villus and the adjacent epithelium is in a stage of necrobiosis. The cellular infiltration of the mucosa is confined to the necrotic villus (fig. 4). The bile contains culturally few bacteria, tinctorially a few clusters may be demonstrated. The focal necroses of the villi resemble the well-known lesions usually found in the liver of the same animals. The mucosa of the fundus is the place of predilection and the tissue destruction may be limited to one or two folds. It is self-evident that serial sections alone will reveal this type of wall infection. Toward the end of the 48th and 60th hour the focal necroses in the villi are in some animals quite numerous and may in certain cases be recognized macroscopically as small yellowish spots. The epithelium of the villi is frequently intact and shows normal nuclei. The photomicrographs (figs. 5 and 6) illustrate the location of the areas either in the top or the base of the villi. The center of the necroses consists of cellular débris, fibrin and

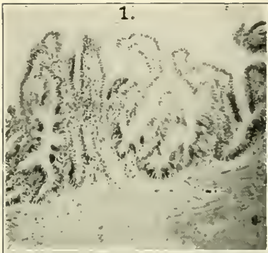
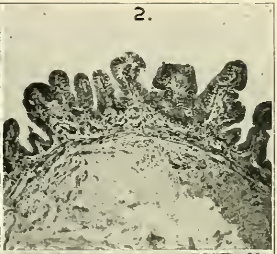
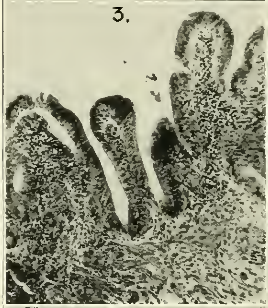
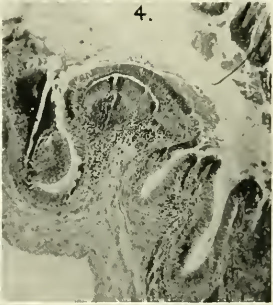
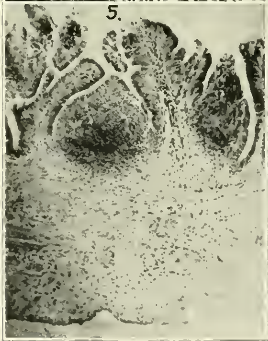
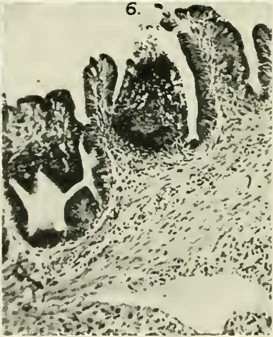







Fig. 1.—Rabbit 725; fundus of normal gallbladder.

Fig. 2. Six hours. Rabbit 1701 A; intravenous 8 billion; green bile, sterile; wall innumerable colonies.

Fig. 3.—Twenty-four hours. Rabbit 1704. Direct gallbladder injection; 22 billion typhoid bacilli; colorless, limpid fluid with leukocytes, no blood.

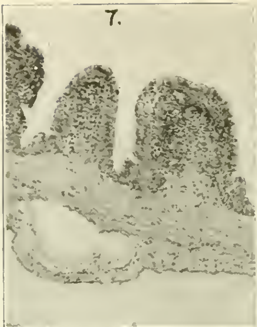
Fig. 4.—Twenty-four hours. Rabbit 1707 (11). Six billion intravenous; bile deep green, viscid, 150 colonies per c c; wall ∞ colonies; macroscopically intact mucous membrane.

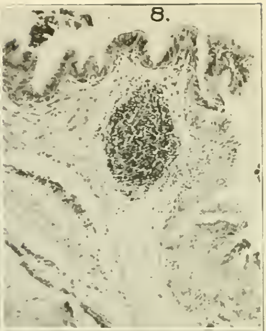
Fig. 5.—Rabbit 897; B. typhosus ½ blood-agar slant; bile deep green viscid, ∞ colonies from bile and gallbladder; subserous edema; mucosa slight erosions.

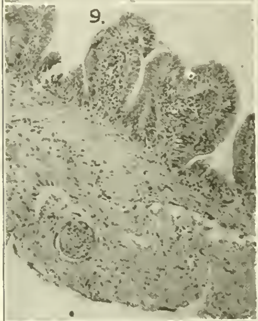
Fig. 6.—Rabbit 897; B. typhosus ½ blood-agar slant; bile deep green viscid, ∞ colonies from bile and gallbladder; subserous edema; mucosa slight erosions

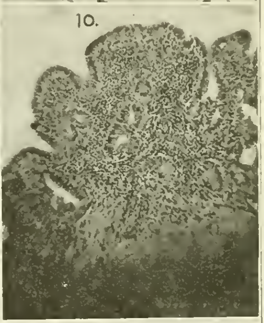
clusters of bacteria. Occasionally the demarcation and the discharge of the necrotic villi into the lumen of the gallbladder can be followed. Culturally at this stage of the infection the cystic bile may show one million bacteria per c c. The presence of a large number of bacilli in the bile leads to an edema and a diffuse cellular reaction of the entire mucosa of the gallbladder, while the localized necrosis may develop into a diphtheritic tissue defect, which extends into the muscularis, submucosa and serosa. In some instances a fibrinous pericholecystitis, even a perforation of the ulcerative, gangrenous inflammation has been observed. The formation of hemorrhages, the focal necroses and the diphtheritic lesions on the tips and at the base of the villi definitely suggest an embolic invasion of the terminal capillaries.

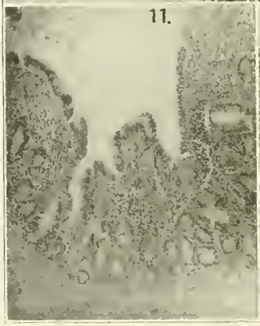
Theoretically it is remotely possible that the typhoid bacilli, which reach the bile by way of the hemato-hepatogenous route, multiply in the stagnant content of the gallbladder and invade the villi from within. Moreover, it is not unlikely that the bacteria can destroy the epithelial lining and enter the mucous coat in consequence of these defects. As the entire conception of the transverse infection rests on the conclusive demonstration of a primary focus in the capillaries of the wall, we have inoculated large doses (1 to 4 billion) of typhoid bacilli in 0.5 c c of saline directly into the gallbladder of normal rabbits in order to determine whether the gallbladder foci described in the foregoing could be developed in a similar manner by this route. The injections have been made with and without ligature of the needle puncture according to the method of Venema.[23] Six animals successfully operated on without hemorrhage into the gallbladder, have been killed 24 to 60 hours after the intracystic injection. Serial sections showed a complete absence of localized areas of necrosis in the villi. The histologic picture (fig. 3) is characteristic for a catarrhal cholecystitis. The epithelium is intact, invariably studded with emigrating leukocytes, and sometimes it is loosened from the mucosa. The latter is diffusely infiltrated with lymphocytes and polymorphonuclear leukocytes. The inflammatory process involves the lymphatic spaces and lymph vessels of the submucosa and serosa; in fact, the entire gallbladder wall is macroscopically edematous. The dilated lymph vessels and capillaries contain thrombi of leukocytes and lymph material. The lymphostasis resulting from the involvement of the lymphatic system extends along the bile duct into the portal tissues of the liver. Neither necrosis of the tips of the villi nor diphtheritic ulcerations analogous to illustrations 4, 5 and 6 can be detected. The typhoid bacilli and their products invade the wall











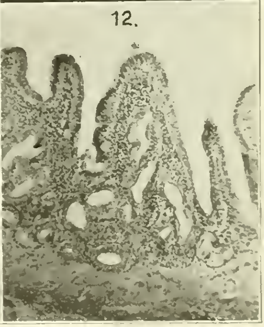


Fig. 7.—Rabbit 1021, 7 days, immunized, 10 billion agglutinated B. typhosus intravenously; gallbladder distended by colorless slimy pus, no erosions; extensive edema along extrahepatic ducts.

Fig. 8.—Rabbit 1095, 8 days, polyhomogenous B. typhosus, gallbladder distended, yellowish nodules and diphtheritic patches on mucosa; bile limpid, colorless. B. typhosus in all the organs.

Fig. 9.—Rabbit 1082, 31 days, 3 injections B. typhosus of recent isolation; loss in weight; intermittent shedding; irregular thickening of gallbladder wall, bile viscid, light green. B. typhosus in all the organs except heart blood.

Fig. 10.—Rabbit 1072, 32 days, polyhomogenous mixture of B. typhosus I. V.; intermittent shedding, cholangitis, empyema of distended thick walled gallbladder; B. typhosus in bile and wall, liver and duodenum.

Fig. 11. Rabbit 705, 46 days; paratyphosus A, stool continuously negative; contracted gallbladder with 4 greenish soft stones size of a large pinhead; greenish watery bile. B. typhosus in bile and middle lobe of liver, duodenum.

Fig. 12. Rabbit 15 A, 149 days, polyhomogenous I. V.: intermittent intestinal discharge of typhoid bacilli; gallbladder distended, [illegible] c.c. of light green bile with biliary sand about [illegible] of fluid. B. typhosus in bile and urine

by way of the lymphatics; the resulting reactions involve these systems and not the blood vessels. Culturally, the bile invariably contains more typhoid bacilli than the gallbladder wall.

In studying this diffuse reaction it has been noted that the microscopic changes are the same whether the cystic bile has been infected by a direct injection or whether the bacilli enter the bile by way of the hemato-hepatogenous route and multiply in the bile. Moreover, the diffuse inflammatory reaction which follows the transverse embolic wall infection is of the same character. On the 3rd to 8th day after an intravenous injection of a large number of typhoid bacilli it is usually impossible to state from the study of a few sections whether the bacilli reach the gallbladder through the bile or through the blood vessels of the wall. In either case the bile in the viscus has become, on account of the addition of pus, lymph and blood fluids, an excellent substratum for the multiplication of the typhoid bacillus and invariably contains more germs per 1 c c than the wall. Their presence reacts on the layers of the wall in a manner absolutely identical with the one described for the intracystic injection. These facts explain in part the reports of Weinfurther,[58] Nichols [107] and others, who failed to observe the necroses, the diphtheritic inflammation of the villi, and the mechanism of the blood vessel injury which lead to the cholecystitis on account of their failure to make their histologic examinations sufficiently early.

The cellular reactions which develop in the course of the infection of the gallbladder wall are variable; they are usually diffuse, but may occasionally be localized in form of mural abscesses. The importance of these lesions on the persistence of the infection will be treated in a subsequent paragraph, but attention must be called at this point to a section of a gallbladder which was removed from a rabbit on the 30th day after infection (fig. 9). The connective tissue in the submucosa is increased, the blood vessel walls are either thickened and infiltrated with small lymphocytes or they exhibit organizing thrombi, all evidences of what is apparently a chronic inflammatory process. The origin of these extensive blood vessel lesions and their ultimate fate are difficult to determine in that we have not been afforded the opportunity of studying them at varying periods of their development. A systematic search will probably produce additional evidence to support the fact that the infection of the gallbladder wall plays an important rôle not only in streptococcic (Rosenow [67]), but also in typhoid and paratyphoid cholecystitis.

The typhoid bacillus reaches the gallbladder in human cases of typhoid regularly after the liver has been disabled by the poisonous products of the bacteria, and its presence there is evidenced by positive cultures or by a mild or moderately severe catarrhal cholecystitis (E. Fränkel,[108] Posselt[109]). According to the findings of numerous pathologists, the lesions present in the gallbladder and liver are certainly not as severe is frequently assumed by bacteriologists and epidemiologists. Purulent, necrotizing, inflammatory processes are infrequent and the formation of typhoid foci in the gallbladder wall supposedly of hematogenous origin are exceptionally rare. A general application of the conclusions of J. Koch[110] based on an exceedingly severe and unique form of typhoid cholecystitis is by no means justified.

From our own limited number of observations[111] and the evidence which we have been able to gather from the literature (Bindseil; Messerschmidt[112]; Goebel; Nichols, Simmons and Stimmel[113]; Küster, and Günzel[114]), it is evident that the embolic infection of the gallbladder wall plays an insignificant rôle in the human carrier state. A condition analogous to that found in man apparently exists in the guinea-pig. The microscopic and cultural study of a limited number of gallbladders derived from infected guinea-pigs convinces us that the wall is rarely, and then only slightly, infected through the bile. In fact, properly immunized guinea-pigs never develop gallbladder infections.

The factors responsible for the hematogenous infection of the gallbladder of the rabbit have not been studied with the desired thoroughness on account of the large number of animals required for such tests. It has been stated in connection with our work on paratyphoid B infections in these animals, that intravenous or intraportal injections are prerequisites for the development of a cholecystitis. Feeding or subcutaneous injection fails to cause an infection. Similar observations were made by Fränkel and Much[69] with a specifically elective paratyphoid bacillus. In experimental typhoid the individual susceptibility of the rabbits has interfered seriously in the numerous tests undertaken to determine some of the factors which lead to a wall infection. Typhoid strains which produce extensive liver necroses, provoke, as a rule, similar lesions in the gallbladder, provided the inoculated dose of bacteria is sufficiently large and made by intravenous, arterial or intrasplenic injections. Agglutinated bacteria or those grown on potatoes or blood agar give positive results more frequently than those cultivated in broth. Furthermore, starvation and changes in diet appear in some of the experimental series to be the factors conducive to wall infections.

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[108] Mit. a. d. Grenzgeb. d. Med. u. Chir., 1909, 20, p. 898.

[109] Ergebn. d. allg. Path. u. path. Anat., 1919, 19, pp. 351 and 471.

[110] Ztschr. f. Hyg. u. Infekionskrankh., 1908-09, 62, p. 1

[111] We examined several gallbladders of convalescent typhoid patients which had been removed on account of gallstones. Histologically a diffuse infiltration of the mucosa and submucosa, or nests of round cells covered by an intact epithelium were observed. The cultural findings in one case also support the anatomic findings that the wall is not seriously involved, namely, the bile contained 30,000,000 typhoid bacilli per c.c. while 1 gm. of washed wall contained 240 bacilli.

[112] Ztschr. f. Hyg. u. Infektionskrankh., 1913, 75, p. 411

[113] Jour. Am. Med. Assn., 1919, 73, p. 680.

[114] Ztschr. f. Hyg. u. Infektionskrankh., 1916, 81, p. 447

THE FACTORS RESPONSIBLE FOR THE PERSISTENCE OF THE TYPHOID BACILLUS

Some writers on experimental cholecystitis conclude from their studies that the gallbladder is the only site in which the typhoid bacillus persists long after it has disappeared from all other parts of the body. These conclusions are based on a limited number of experimental animals and on incomplete tissue cultures. The recent reports of Hailer and Ungermann,[105] of Wagner and Emmerich,[115] fail to support this view. The first named writers found the spleen infected in 56%, the liver in 66%, and the kidneys in 46%; while the gallbladder or its contents harbored the typhoid bacillus in 93% of the instances. The data of Wagner and Emmerich demonstrate even more conclusively the participation of other organs as sites for the persistence of the bacilli in the rabbit. Animals killed on the 226th or 346th day harbored typhoid organisms in the spleen, lungs, liver and gallbladder. We collected at random the necropsy findings of 18 rabbits, which were killed or had succumbed to an intercurrent infection on the 64th to the 816th day after an intravenous inoculation. These animals were chosen because minutely detailed cultures were made on their tissues. The findings are shown in table 4. Additional data will be published in subsequent papers.

It is evident from table 4 that animals which succumb to intercurrent infections (due to B. cuniculisepticus, enteritis) or perhaps auto-infections, may exhibit an extensive dissemination of typhoid bacilli in the various viscera. Rabbits, on the other hand, which were in good health when killed, usually showed typhoid bacilli only in

[115] Med. Klin., 1916, 12, p. 819.

TABLE 4

Anatomic and Cultural Findings in Chronic Typhoid Infections of Rabbits

Rabbit	When Killed After Infection	Anatomic Findings on Gallbladder	Bacteriologic Findings												
			Bile	Gall-bladder Wall	Liver	Spleen	Bone-marrow	Kidneys	Urine	Mesenteric Lymph Nodes	Lungs	Heart Blood	Duodenum	Jejunum	Ileum
840	180 days	Small, thick wall, bile light green with granular calculi......	T	T	0	0	0	0	0	...	0	0	0	0	0
884	81 days	Distended, thick, light blue, granular sediment about ⅓ calculi	T	T	0	0	T	0	0	...	0	0	T	T	T
893	245 days	Distended, cystic duct blocked, light green with blackish calculi	T	T	T	0	T	0	0	T	0	0	0	0	0
10	99 days	Distended, thick wall injected, light green bile with course granular sediment	T	T	T	0	0	0	0	0	0	0	T	T	T
13	103 days	Distended, very thick wall, light green bile with coarse granlar sediment	T	T	0	0	0	0	0	0	0	0	0	0	0
19	103 days D. Pneumonia and pericarditis	Distended, very thick wall, purulent whitish with sediment...	T	T	T	T	T	T	—	T	—	T	—	T	T
800	83 days	Typical thick wall and light green bile, heavy sand-like débris	T	T	T	0	0	0	0	T	...	T	T	T	T
805	84 days	Distended, thick wall, light green bile with pus and sandy débris	T	T	0	0	0	0	0	...	T	...	0	T	TT
856	232 days	Thick wall, adhesions, light green bile with blackish calculi..	T	T	T	0	T	0	0	0	0	0	T	T	TT
865	409 days	Thick wall, light green, sand-like sediment with blackish calculi	T	T	0	0	0	0	0	0	0	0	T	T	TT
912	61 days D. pneumonia	Thick wall, purulent flocculent bile, yellowish concretions, extensive cholangitis	T	T	T	T	T	0	0	0	T	0	0	0	0
918	140 days	Thick wall, light greenish bile, sand-like débris............	T	T	T	0	0	0	0	0	0	0	T	0	T
871	102 days D. pneumonia	Thick wall, adhesions, pure pus, and blackish débris, cystic duct obstructed	T	T	T	0	T	0	0	0	0	0	0	0	0
1034	219 days	Thick wall, yellow, slimy purulent bile............	T	T	T	0	0	0	0	0	T	0	T	T	T
946	86 days	Thick wall, clear light green bile, cholangitic abscesses........	T	T	T	0	0	0	0	0	0	0	T	T	T
1067	162 days	Thin walled, dark green............	0	0	0	0	T	0	0	0	0	0	0	0	0
1036	251 days	Small, thick wall, few drops of light yellow-greenish sediment; viscus atrophic, cholangitis of the extra hepatic ducts......	T	T	0	0	0	0	0	0	0	0	T	T	T
717 Para A.	816 days (relapse ?)	Size of a plum, thick wall, blackish granules in purulent light brown (largest gallbladder noted)............	T	T	T	T	T	0	0	T	T	T	T	T	T

the liver, bone-marrow and intestinal content. The persistence of the microbes is therefore not confined to the bile and gall-bladder wall. In one exceptional instance an animal came to necropsy in which the injected organisms were found only in the liver; and in another isolated animal killed on the 162nd day, only in the bone-marrow. Under special conditions, a prolonged localization in the kidneys can be demonstrated. In guinea-pigs a general dissemination and persistence in the tissues is the rule, while gallbladder lesions may be absent although typhoid bacilli are continuously discharged in the stools.

Particular study has been given to rabbits which show foci in the liver and bone-marrow. Thus far typhoid bacilli have not been found in the intestinal tract, bile or gallbladder of animals which carried the infection essentially in the remote viscera. Even liver foci have been recognized culturally, while typhoid bacilli have been absent in the cultures made from the bile and the intestines. In our experience the intestinal discharge of typhoid bacilli in the rabbit is, as a rule, associated with a gallbladder infection. It remains, therefore, to be proved, at least for this animal, that a closed liver focus or any distant focus, provided gallbladder infection is absent, is in a position to infect the intestinal tract through the bile, to which the organisms are supposed to have been carried by the blood or lymph stream. The factors responsible for the persistence of typhoid bacilli in the rabbit are in all probability confined to the gallbladder and to the extra- and intra-hepatic biliary system.

The question, "What is the average period of persistence of typhoid bacilli in the gallbladders of rabbits and guinea-pigs infected by way of the blood stream or by direct injection," must be answered before an analysis of the causes can be undertaken. Most observers who have kept rabbits for periods extending over 30 days agree that the localization of the bacteria is a temporary one in 60 to 70% of the animals. Lemierre and Abrami [47] have noted a restitution of the inflammatory process on the 6th day, while Lentz, Hailer and Wolf [60] have observed recoveries at the end of the second and third week. Morgan [50] found at the end of the 4th month 3 out of 7 animals, and Doerr 1 out of 2 rabbits, free from typhoid bacilli. According to Hailer and his associates,[116] the duration of the infection depended on the degree of the lesions; in the course of 2 to 3 weeks, 40% of the rabbits lost their infection. On direct inoculation into the gallbladder they failed in several rabbits to recover the organisms on the 32nd and 38th days. Similar findings were made by Emmerich and Wagner.[117] Our observations on a series of 131 successfully infected rabbits showed that 85% gave positive cultures on the 26th to 34th day when the bile samples were obtained by gallbladder punctures; 10% of the animals exhibited thickened gallbladder walls, while 5% were macroscopically normal. Infected animals when kept under observation for 80 to 100 days recovered from the specific cholecystitis in 72% of the instances. After a lapse of from 120 to 450 days a series of 35 rabbits showed typical thickened gallbladder walls, but the typhoid bacillus was isolated only from 10 to 27% of the animals. The cultures of several gallbladders gave a growth of indifferent streptococci. Based on these data, it must be concluded that successful typhoidal gallbladder infection in rabbits is frequently a temporary process. Chronic infections comparable with those of the human carrier at the end of 3 months are found only in 10% of the infected rabbits. As a rule, direct intracystic inoculations are conductive to a prolonged persistence (for more than 100 days) in a higher percentage of cases than intravenous injections. This corresponds to the findings of Wagner and Emmerich. Rabbits with chronic infections are always in perfect health and gain in weight. They are occasionally the victims of intercurrent infections, but not to a greater extent than control animals.

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[116] Arb. a. d. k. Gsndhtsamte, 1914-15, 48, p. 80.

It is obvious that these observations in rabbits are in many respects analogous to those in human carriers. It may be mere coincidence that the percentage of 11.6% of typhoid carriers found in India by Semple and Creig[118] corresponds rather closely with the data collected on rabbits. The majority of the rabbits can, following the classification of Sacquépée,[119] be placed in the group of "temporary" or "convalescent" carriers; while 10% develop into "chronic" carriers. It is a well-known fact that typhoid bacilli may persist in the stools of human beings for 3 months after apparent recovery from typhoid or paratyphoid fever, and according to Reibmayr[120] 25% of these convalescent carriers free themselves of the infection spontaneously. Moreover, Goubau[121] has shown that autovaccination clears such carriers of the bacteria much sooner than when they are left untreated.

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[117] Centralbl. f. allg. Path. u. path. Anat., 1916, 27, p. 433; Med. Klin., 1916, 12, p. 74.

[118] Scientific memoir by officers of the Medical and Sanitary Department of the Government of India No. 32, Calcutta, 1908.

[119] Bull. Inst. Pasteur, 1910, 8, pp. 1 and 49.

[120] Munchen. med. Wchnschr., 1918, 65, p. 670.

[121] Arch. Méd. Belges, Paris, 1917, 70, p. 590

It must be borne in mind by every experimenter that spontaneous recovery of the temporary carrier state in rabbits is rather the rule than the exception. These animals are therefore unsuitable for practical chemotherapeutic tests. Chronic carriers, particularly rabbits, infected by direct inoculation of the gallbladder, develop lesions which cannot be treated pharmaceutically and usually present a therapeutic difficulty which is rarely encountered in human cases. Appreciating these limitations, it seems obvious that therapeutic results obtained in rabbits should not be unreservedly applied in drawing analyses as to the possibility of treating similar conditions existing in man. The proper procedure in studying the pharmacologic influence of various substances on typhoid carriers consists in testing them first on dogs with biliary fistulas, followed by experiments on chronic rabbit-carriers infected by the intravenous route. Then human cases, on whom a cholecystotomy has been done and drainage instituted, should be investigated in order that the bile secretion can be tested chemically as well as bacteriologically. Finally the same problems should be studied in chronic human carriers. In all typhoid cases an attempt should be made to prevent by proper treatment (high calory diet instead of starvation, which favors biliary stasis) the development of a carrier state. Carriers should be treated in the earliest possible stage by intensive autovaccination. For this purpose a few chemicals have been suggested, but their value has not been definitely proved. A prolonged carrier state usually leads to severe lesions which cannot be influenced by such procedures. The radical removal of the gallbladder is then not only from a hygienic standpoint, but also on account of the frequent presence of, or tendency toward, gallstones, in the interest of the carrier himself.

Having recognized in the rabbit the occurrence of temporary and chronic gallbladder infections, it is important to determine, if possible, the causes responsible for these variations. In this connection the various routes leading to gallbladder infections deserve further attention. A hemato-hepatogenous elimination of the bacteria is suspected of causing a temporary cholecystitis, while a wall invasion results in a chronic persistent infection. It has been repeatedly stated that the inoculation of several billion typhoid bacilli is necessary to produce an experimental gallbladder infection. In case this particular prerequisite is not fulfilled, neither a discharge of bacilli through the bile nor an embolic invasion of the wall takes place. It is known from our own observations and those published by others, that the bacterial elimination in the bile is temporary and, as a rule, in the first 6 hours comparatively few typhoid bacilli reach the bile in the gallbladder, where unhindered multiplication can ensue. The few organisms which enter the bile are probably rapidly discharged into the intestines, as is evidenced by the findings in spontaneously and artificially produced (feeding) paratyphoid B infections (Litch and Meyer[43]). In case focal lesions, which communicate with the biliary passages, are established, as can be proved by intrahepatic injections (Hailer and Rimpau, personal observations), the elimination of large numbers of typhoid bacilli may be continuous for several days. Injury of the liver is accompanied by intoxication, with numerous physiologic and metabolic disturbances, one of which is biliary stasis. The bacilli reaching this viscus may remain, therefore, a sufficiently long period to permit of multiplication in a medium excellently suited for this purpose. In turn, the inflammatory reaction of the mucous membrane contributes an admixture of cells and lymph material, which favors the growth of the organisms in the cystic bile. Anatomically a catarrhal cholecystitis with little or no involvement of the submucosa and muscularis may be recognized. In these cases cultures of the bile give innumerable colonies of B. typhosus, while the washed wall is nearly sterile. This development of a mild gallbladder infection can be readily studied by direct inoculation of a small number of typhoid bacilli into the gallbladder. The degree of inflammation is moderate and the process involves mainly the subepithelial lymph system of the mucosa with a temporary lymphostasis in the adjacent systems of the muscularis and subserosa (fig. 7). Rabbits harboring such gallbladders are in the group of temporary carriers. At necropsy on the 10th to 20th day one finds a normal sized gallbladder with a slightly thickened wall which appears rather light in color. The bile is light or deep green, limpid, but contains no concretions of mucus. The P^{+}_{H} of the bile is less than 7.2 and changes rapidly to P^{+}_{H} 8.0-8.6. Cultures give typhoid colonies varying in number from 10 to several hundred per loop of bile. A careful histologic study of a large number of such gallbladders invariably shows an intact epithelium with lymphocytic infiltration of the mucosa, moderate proliferation of the connective tissue of the submucosa and a well preserved muscularis; no primary wall infection can be detected. The anatomic evidence supports the contention that these types of subacute cholecystitis may heal in from 2 to 4 weeks and then terminate the carrier state.

The etiology of the prolonged, temporary (50 to 100 days) or the chronic carrier state cannot be determined with certainty from our material. However, certain factors conducive to the persistence of the infection and the chronicity of the process can in a number of cases be definitely recognized. Diphtheritic foci of the gallbladder mucosa discharging clusters of typhoid bacilli into the bile are usually associated with liver lesions. The bile is being seeded from these numerous extensive foci. The inflammatory reaction resulting from this heavy and continuous infection is most severe as can be seen in gallbladder specimens removed on the 6th to 10th day of the disease. In exceptional instances the wall may show deep-seated abscesses (fig. 7). Usually the mucosa is partially necrotic, covered with leukocytes and fibrin; the submucosa and serosa are infiltrated with cells and fibrin and may even show small and large hemorrhages. The intrahepatic bile ducts not infrequently exhibit a marked inflammatory reaction. The bile is purulent, quite often blood tinged. These extensive changes may lead to adhesions or in exceptional cases to perforation. It is quite evident that such a profound involvement of the biliary system produced various anatomic and physiologic conditions, which favor the continuation of the infection. In chronic rabbit gallbladder carriers one of two findings is quite constant, namely, (1) empyema of the viscus with severe inflammation of the wall or (2) biliary sand or small calculi. The macroscopic findings of a number of gallbladders are reported in table 4. Histologic studies have been made on fresh tissue. Specimens with denuded mucosa are of questionable value and have not been used. The main findings are briefly as follows:

The outstanding features of the microscopic picture of the gallbladder showing an empyema is the thickening of the mucosa and submucosa to 4 to 8 times its normal dimension. The papillae of the mucosa are stumpy and diffusely infiltrated with lymphocytes. Occasionally nodular areas resembling lymph follicles can be observed. The covering epithelium exhibits a marked hyperplasia, but is intact. The cellular infiltration extends to the muscularis, which is either atrophic or its oblique fibers are increased in numbers (fig. 10). The stroma of the fibrous serous coat is enormously thickened and permeated with nests of lymphocytes and leukocytes. Frequently the wall of the blood vessels is thickened and infiltrated. The inflammatory process involves the cystic duct and frequently the extra- and intra-hepatic biliary system. A distinct cholangitis is found in 50% of the rabbits killed between the 50th and 100th day of the infection. The bile is stringy, purulent and colorless. On standing, leukocytes and some biliary sand are sedimented (from 1/2 to 2/3 of the fluid bulk).

The gallbladder of the animals examined after the 30th day of the infection and that of every rabbit which harbored the typhoid bacilli for more than 100 days exhibit additional noteworthy microscopic changes. The thickness of the wall is not materially increased, but the mucosa exhibits elongated proliferations of the papillae. The entire mucosa coat contains numerous glands, which frequently show papillomatous extensions into the muscularis and submucosa resembling an adenomatous growth. Hypersecretion of mucus is distinctly visible, (figs. 11 and 12). An intact but hyperplastic epithelium covers a diffusely cellular-infiltrated stroma. The connective tissue growth in the serous and even the muscular coat is extensive; the lymph cell infiltration is comparatively slight. Round cells are, however, frequent in the hepatic connective tissue. The bile is rather viscid, the P^{+}_{H} is above 7.8, contains either a large amount of coarse, sand-like biliary concretion or even one or several well formed facetted stones. The latter may be black and friable. The findings are characteristic of those found in chronic cholecystitis. Invariably cultures prepared from the bile, stones, and wall give abundant colonies of B. typhosus. It has been impossible to demonstrate conclusively by tinctorial methods the bacilli in the tissues.

About 40% of our carriers killed before the 100th day exhibited the lesions of an empyema of the gallbladder, which explains readily the persistence of the bacteria in the biliary system. The empyema develops in consequence of an obstruction of the cystic duct or from a loss of contractibility of the wall. The latter may follow or precede the obstruction and is probably caused by the extensive proliferation of the connective tissue, submerging the important longitudinal and oblique muscle fibers. According to the available notes, cultures from the duodenum are frequently positive. Evidently the bacteria are eliminated with the hepatic duct bile from certain intrahepatic foci, because the inflammation in the cystic duct is sometimes so profound as to prevent a discharge of the gallbladder contents. These hepatic areas of necrosis or the microcholangitic abscesses are probably analogous to the lesions described by Blachstein,[7] and they explain the occasional persistence of typhoid bacilli in the stools of rabbits after cholecystectomy which does not always cure intestinal carriers (Loele[122]; in exper. 1 and 2).

Exper. 1.—Rabbit 1625, weighing 2,175 gm. was inoculated on Aug. 8 and 9 1918, with 1 mg. of uranium nitrate, subcutaneously. On Aug. 20, 1918, it received an injection of 1,500 million B. typhosus, grown on potatoes, directly into the left kidney. The stool and urine were positive on Aug. 29, Sept. 2, 11, 15, 23 and Oct. 1. On Oct. 3 a complete cholecystectomy of an enlarged typical gallbladder was performed. The stool was negative on Oct. 4, 5, 6, 8, 9, 15, 25 and Nov. 10; while the urine was positive on Oct. 4, 5 and 8, and negative on Oct. 9, 15, 25, 30, and Nov. 10. At necropsy, Dec. 24, 1919, all the organs were found sterile.

Exper. 2.—Rabbit 1630, weighing 2,175 gm., was inoculated Oct. 8 and 9 with 1 mg. of uranium nitrate, subcutaneously. On Aug. 20, 1918, an injection of 1,500 million typhoid bacilli grown on potatoes was made directly into the left kidney. The urine was positive on Aug. 23, 29, Sept. 8, and Oct. 1. The stool contained bacilli on Aug. 29, Sept. 8, and Oct. 1. A complete cholecystectomy was performed on Oct. 3, 1918. The gallbladder was typical; the bile contained 3,200,000 bacteria per c c and the wall 3,500,000 per 100 mg. Recovery from the operation was rapid and complete. Urine cultures remained negative, while the stool cultures were positive on Oct. 4, 5, 6, 8, 15, 22, 28, 30, Nov. 10, and then remained negative on daily examination. At necropsy on Dec. 21, or 78 days after the operation, B. typhosus was isolated from the liver, the left kidney, the left bone marrow and a small abscess between the stomach and the liver. The intestinal tract was negative for B. typhosus.

From these experiments it is evident that the removal of the gallbladder does not always cure the carrier state, although the observations are not sufficient to warrant absolute deductions. In this respect the findings are analogous to those reported for human carriers, namely, cholecystectomy does not always cure intestinal carriers (Loele;[122] Fromme;[123] Schultze;[124] Nichols, Simmons and Stimmel[107]).

The bile of "chronic carriers," that is, rabbits which remained infected 100 to 816 days, contained invariably yellow-greenish biliary concretions, or blackish calculi. It appears of interest to consider briefly the nature and origin of these stones. The small concretions are microchemically composed of lime salts, traces of bile pigment and organic material, but no cholesterol. They are "Entzündungsteine" in the sense of Aschoff and Bacmeister,[125] or calcium concretions, which develop as a result of the inflammatory process. It is well known and has been repeatedly shown that a chronic cholecystitis leads to an hyperplasia of the mucous glands and to an excessive production of biliary mucus. Aschoff believes this mucus to be very rich in lime salts. Lichtwitz and Bock[126] consider the calcium content of the bile to be normal. Its precipitation, however, is facilitated by the excessive and concentrated glandular secretion.

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[122] Deutsch. med. Wchnschr., 1909, 35, p. 1429.

[123] Deutsch. Ztschr. f. Chir. 1910, 107, p. 578.

[124] Centralbl. f. d. Grenzgeb. d. Med. u. Chir., 1913, 49, p. 1892.

[125] Die Cholelithiasis, Jena, 1909

[126] Deutsch. med. Wchnschr., 1915, 41, p. 1215.

Irrespective of the uncertainty relative to the origin and the physical-chemical factors which lead to the precipitation of the calcium salts of the bile, it has been definitely proved (Rosenbloom [127]) that biliary calculi composed of lime are regularly due to an infectious process. The conditions favorable for the development of such stones are unquestionably present in the chronic cholecystitis of the rabbit. Calculi once formed favor the perpetuation of the inflammatory process and with it the persistence of the typhoid bacillus. The precipitated calcium salts are in part also responsible in carrier rabbits for the well-known low H-ion concentration of the cystic bile exposed to air for a few minutes. The large black or greenish stones have, in our opinion, a different origin. They are soft and friable; chemically they are composed of protein material (30 to 60%), some bile pigment and at times

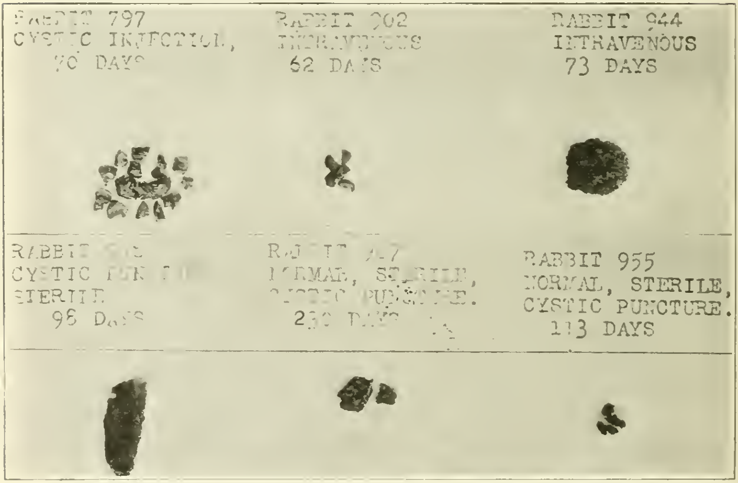

Fig. 13.—Stones (actual size) originating in gallbladders of rabbits inoculated intravenously with typhoid bacilli.

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[127] Jour. Am. Med. Assn., 1917, 69, p. 1765.

traces of lime salts. Certain stones (example shown, natural size, in fig. 13) originate in the gallbladders of rabbits which have been inoculated intravenously with typhoid bacilli. They are likewise found in this viscus after a direct inoculation by puncture of the gallbladder. It has been noted that blood clots develop quite frequently subsequent to a needle puncture or the application of a ligature. Hemorrhages and blood extravasations also result, in intravenously inoculated rabbits, as the consequence of the diphtheritic, necrotizing cholecystitis. The albuminous, organic nucleus of these stones is therefore primarily one of these blood coagulums, which in some cases may secondarily become impregnated with calcium salts (fig. 13, rabbit 944). It is obvious, that absorption of this type of stone is practically impossible and their presence assists in the prolongation of the cholecystitis and the persistence of the infection. This interpretation probably applies to the frequent findings of biliary calculi of this type in the gallbladders of rabbits infected by direct cystic injections (Emmerich and Wagner).

The concretions ordinarily found in rabbits should not be compared with the cholesterol stones of man. This fact has been emphasized by Klinkert,[18] who repeated the experiments of Cushing,[128] in which the gallbladder was first injured by compression with forceps, and the animal subsequently infected by an intravenous injection of typhoid bacilli. Bilirubin-calcium calculi could be found on the 54th day after the operation. Pure cholesterol stones artificially placed in the gallbladders of rabbits are, in our experience, gradually dissolved. By feeding cholesterol or lanolin we succeeded in producing pure cholesterol calculi, as will be discussed in paper VI of this series. Biliary calculi have been observed occasionally in rabbits infected with typhoid or colon bacilli and cholera vibrios (Richardson [1] Cushing;[128] Doerr;[1] Forster and Kayser;[46] Creig [84]). Spontaneous stone formation without previous bacterial injury of the gallbladder wall has not been reported. The absence of calculi in the rabbits killed before the 30th day (our own observations are supported by those of Hailer and Rimpau) and their constant presence in animals necropsied after the 100th day are significant. It justifies the conclusions that the chronic inflammation provoked by the typhoid bacillus is the cause of the precipitation of the lime salts. The resulting calculi in turn are probably the main factors favoring the persistence of the bacteria in the gallbladder.

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[128] Bull. Johns Hopkins Hosp., 1899, 10, p. 166.

These findings indicate that the persistence of the typhoid bacilli in the rabbit's gallbladder is quite analogous to the carrier state in man. This latter condition is, according to the observations of Nichols, Simmons, and Stimmel,[113] of Messerschmidt,[112] of O. Mayer,[129] and others, associated in 65 to 90% of the cases with gallstones, while the remaining 10 to 35% of human carriers invariably show a distinct chronic cholecystitis or an empyema of the gallbladder. The excellent summaries of Posselt[109] and of Wagner and Emmerich[15] supply additional information for this comparison and should be consulted by those interested in the subject of cholecystitis caused by typhoid fever.

In the preceding discussion the function of the bile has not been considered as a factor controlling either the development or the persistence of a carrier state in rabbits. Comparatively recently Nichols expressed the opinion based on test-tube experiments that the biles of certain rabbits possess an inhibitory or even an antiseptic action. Our detailed studies reported in paper VI, VII and VIII, fail to support this contention. In fact, it is proved that the cystic bile of rabbits is particularly suited for the growth of B. typhosus.

TABLE 5

AGGLUTINATION OF TYPHOID BACILLUS IN CARRIERS AND RECOVERED CARRIERS

Carrier		Recovered Carriers	
99 days	1:1,000	134 days	1:2,000
103 days	1:1,000	149 days	1:2,000
140 days	1:2,000	180 days	1:2,000
180 days	1:2,000	205 days	1:600
232 days	1:600	252 days	1:100
245 days	1:4,000	314 days	1:600
409 days	1:400	430 days	1:200

A comparative analysis of a limited number of serologic tests fails to suggest any relation between demonstrable immune bodies and the carrier state. This should be expected in the light of the data discussed in paper IV. The serum of chronic carrier animals agglutinates the typhoid bacillus in dilutions above 1:100 quite frequently in dilutions as high as 1:4,000. In comparison with the original titer determined 15 to 20 days after the infection, the agglutinating power of the serum is considerably diminished. After a lapse of 100 to 200 days the immune bodies remain, however, fairly constant. Recovered carriers differ in no respect from the animals harboring a focus of infection, as is illustrated by the figures in table 6. Complement fixing antibodies

[129] Munchen. med. Wchnschr., 1914, 61, p. 1116

disappear more rapidly than the agglutinins, but no differences are noted between the infected and the recovered animals. It is well known that in rabbits the disappearance of agglutinins following an infection is fairly rapid in the first 2 to 3 months, and may remain constant for over one year. Guinea-pigs behave differently; such animals harboring typhoid bacilli in their tissues may give negative Widal reactions on the 50th or 60th day after infection. It is evident that serum tests can neither be used to detect a carrier state in rabbits or guinea-pigs, nor can they explain the recovery or the persistence of the bacilli in the tissues and particularly the gallbladder. Experiments conducted several years ago have proved that cutaneous hypersensitiveness tests are more marked in infected than immunized rabbits. Skin tests may therefore be employed to advantage in the detection of the rabbit carrier state.

In conclusion, it can be stated that the temporary typhoid carrier state in the rabbit is probably the result of a hemato-hepatogenous infection of the bile. The resulting inflammation of the gallbladder is comparatively mild and a restitutio ad integrum can take place readily. The chronic carrier state is favored by a severe wall infection resulting from an embolic invasion of the terminal capillaries of the mucosa. The persistence of the typhoid bacillus is facilitated by an obstruction of the cystic duct, the development of an empyema and the formation of biliary calculi. These chronic infections can be produced more readily in rabbits resistant to the typhoid toxin or by the injection of an atoxic strain in doses of not less than 2 billion per kilogram.

GALLBLADDER INFECTIONS PRODUCED BY THE INOCULATION OF TYPHOID BACILLI INTO THE SPLEEN AND INTO THE GALLBLADDER CONTAINING GALLSTONES

Hailer and Ungermann[105] attempted by direct inoculation of B. typhosus into the liver, kidneys, duodenum, appendix and gallbladder to produce typhoid foci in which the bacilli were protected from the destructive influence of the blood serum (?). It was also expected that these areas would constantly reinfect the other tissues of the body and favor a more chronic infection than could be obtained by intravenous injections. With the exception of the direct inoculations into the gallbladder and perhaps the liver and kidney, neither of the procedures offered any advantage over the usual intravenous method. The injections into the intestinal tract gave inconstant results, while the liver injections provoked extensive necroses which had nothing in common with the lesions found in man. Our experience with these methods was similar.

The explanation of the carrier state by Webb-Johnson[8] already referred to, suggested the advantage of direct inoculation of typhoid bacilli into the spleen. This is, however, not the only reason why this method has been tried. There are many arguments which may be advanced against the intravenous injections. In the first place, there is necessarily considerable dilution of the infective material and an extremely good opportunity for the operation of any deleterious action which the plasma and cells may possess. Moreover, it seems logical to introduce the infective agent into the locations which are involved in the spontaneous infections as exemplified in the experimental production of tuberculosis and syphilis by inoculation into the testes. The spleen is readily exposed by laparotomy under ether anesthesia, and subcapsular injections of 0.5 to 1.0 c c can be made without injury to the organ.

Thus far we have practiced this method on 16 large rabbits (average weight 2,240 gm.). They have all recovered from the operation; when killed 11 to 31 days after the injection, 8 animals harbored typhoid bacilli in the gallbladder, spleen, liver, bone-marrow and intestines. The intoxication has been slight in comparison to that of the intravenously injected animals. In this connection it should be emphasized that the number of typhoid bacilli introduced did not exceed 500 million. It is well known that this dosage of bacilli produces gallbladder lesions only in exceptional instances in small rabbits. The remaining 8 rabbits are still under observation and are apparently infected, judging from the presence of the typhoid bacilli in the stools. The local lesions produced by the infection are slight; a few adhesions of the splenic capsule to the peritoneal wall, a minor spleen tumor and a small area of necrosis are the only changes recorded on the 31st day. From an experimental standpoint this method is an excellent one and a high percentage of carriers with a low initial mortality can be produced. These results naturally incline one to accept the view of Webb-Johnson. The spleen of the human carrier may be the focus which continuously supplies the bile with the specific organisms. Typhoid bacilli have been found in the spleen of the majority of chronic human carriers, examined after death. Their presence has been attributed to auto-infection, but some of the patients unquestionably died from pneumonia, apoplexy (Günther and

Böttcher,[130] Kamm[131]), without symptoms of auto-infection. In rabbits, however, a chronic gallbladder infection has never been found to be associated with a splenic focus. In our experience the only positive spleen cultures obtained were made from animals which died from intercurrent diseases (table 4, 19, 64, and 816). It is, however, possible that the direct splenic inoculation, which reproduces the conditions in man more accurately than the intravenous method, may furnish conclusive support to the above theory. Such information can, however, only be procured from animals which have been kept under observation for several years. A final opinion concerning this interesting conception of the carrier state by Webb-Johnson will be rendered when the studies of the remaining 8 rabbits are completed.

There had been considerable discussion as to whether the typhoid bacillus is capable of exciting the formation of gallstones or whether the stones are usually preformed, and as such predisposed to a localization of the invading organism. Having satisfactorily demonstrated the lithogenic properties of the typhoid bacillus in the gallbladder of rabbits, it is of interest to test experimentally the second view. The experiments conducted for this purpose are not entirely satisfactory. The introduction of a stone invariably leads to a secondary invasion of the gallbladder wall or the bile by "indifferent" intestinal streptococci. Under these circumstances the viscus is predisposed to a subsequent infection and typhoid bacilli as well as streptococci thrive in the tissues. In some experiments the typhoid bacillus is isolated from the gallbladder wall, while the bile itself contains streptococci. Moreover, it is proved that a relatively small dose of bacteria may localize in the gallbladder in which a calculus has been previously placed. Two experiments of a series of 8 are presented below to illustrate the above statements.

Exper. 1.—Rabbit 1399, weighing 3,095 gm., was laparotomized on March 3, 1919, and a fragment of a sterilized cholesterol stone removed from a human typhoid carrier was placed in the gallbladder. On June 9, 1919, or 97 days after the operation, the animal was killed. The gallbladder was distended and filled with pus-like yellowish fluid containing small fragments of the inserted stone and fine sand-like débris. The wall was thick and fibrous, the mucous coat was injected and partially denuded. The serous coat was edematous and the periportal lymph nodes were soft and edematous. The other organs were normal. Streptococcus salivarius was isolated from the bile, gallbladder wall, regional lymph node and duodenum.

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[130] Ztschr. f. Hyg. u. Infektionskrankh., 1911, 68, p. 439.

[131] München. med. Wchnschr., 1909, 56, p. 1011.

Exper. 2.—Rabbit 1323, weighing 3,675 gm.; on Nov. 8, 1918, a sterile stone the size of grain was placed in the gallbladder. On Dec. 27, 1918, the animal weighed 3,750 gm., and was injected intravenously with $\frac{1}{100}$ slant or 400,000,000 typhoid bacilli. On Jan. 20, 1919, the rabbit had diarrhea and appeared sick. It was chloroformed. At necropsy extensive adhesions between the liver, stomach and omentum were found. The gallbladder wall was thick, and contained about 3 c c of turbid, viscid, brownish-yellow bile. The stone was partially disintegrated and pigmented. The mucosa was smooth; no erosions were visible. Spleen and lymph nodes were enlarged, and soft; a moderate catarrhal enteritis was present. The bile contained streptococci, while the gallbladder wall, the liver, right kidney, spleen and lung gave pure cultures of B. typhosus. The streptococcus grew in opaque, grayish-white staphylococcus-like colonies.

From these observations it becomes evident that streptococci regularly invade a gallbladder injured by an operative manipulation or when irritated by a calculus. The origin of these bacteria is probably intestinal and the route is a lymphogenous one. A subsequent typhoid bacillus invasion is unable to dislodge these streptococci, but fosters a prolonged sojourn and wide dissemination of B. typhosus in the body. According to Rosenow,[67] Brown,[132] Starr and Graham,[133] and others, streptococci are frequently found in acute and subacute cholecystitis of man. The foregoing data suggest a method to reproduce these infections experimentally. In our experience streptococci may replace a colon or typhoid infection in the gallbladder of a rabbit; it is therefore recommended that extreme caution be exercised in the interpretation of the presence of streptococci as an apparent selective affinity for the gallbladder. In order that this type of experiment may be of some clinical value it is advisable to extend the period of observation over several years. It is not unlikely that such studies may assist materially in an understanding of gallbladder disease of man.

SUMMARY AND CONCLUSIONS

The elimination of typhoid bacilli in the hepatic duct bile of normal and immunized animals has been studied on rabbits, guinea-pigs and dogs provided with temporary common duct fistulas. These experiments have demonstrated that more bacteria appear in the bile of normal rabbits inoculated intravenously with 8,000 to 24,000 million typhoid bacilli than in that of immunized animals of the same litter, provided the last inoculation of the vaccine is administered 20 to 30 days previous to the infection.

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[132] Arch. Int. Med., 1919, 23, p. 185.

[133] Ann. Surg., 1918, 68, p. 188

The elimination of the bacteria by the hemato-hepatogenous or descending route is immediate. The maximum number of colonies develop on the plates prepared with bile specimens collected between the first 5 to 15 minutes following the injection. In subsequent periods the number decreases rapidly and the discharge of bacteria may cease completely at the end of one hour. The ability of the individual rabbit to eliminate bacilli in the bile varies considerably. Even repeated inoculations of large doses (10 to 15 billion) may in a small number of rabbits never lead to a discharge of typhoid bacilli in the hepatic duct bile.

The transit of the bacilli from the hepatic blood vessels to the biliary capillaries is probably governed by the phagocytic action of the endothelial cells. Immunization of the rabbits prevents, to a certain degree, the passage of bacilli. In the immunized guinea-pig the endothelial barrier is exceedingly efficient and an intravenous injection of less than 100 million typhoid bacilli usually gives sterile bile cultures, while in normal guinea-pigs several hundred organisms are eliminated in the fistular bile following similar injections.

Rabbits which have been inoculated with dead or living typhoid bacilli and which have been injectetd intravenously with living typhoid bacilli on the 6th to 10th day after the last immunizing injection, discharge more bacilli than the normal control animals of the same litter. This exceptional behavior of the immunized rabbit is probably due to an incomplete restitutio ad integrum of the injured vascular endothelium. Complete recovery from bacterial vaccination, which evidently does not take place before the 10th day, as a rule prevents the transit of bacilli from the blood to the bile.

The excretion of typhoid bacilli in the hepatic duct bile of dogs is irregular and not definitely influenced by immunization. Injections of less than 10 billion bacilli in dogs varying in weight from 14 to 26 pounds fail to cause an elimination of bacteria.

The removal of bacteria from the circulation in the first 10 to 60 minutes may be the result of an in vivo agglutination, the action of the blood platelets, or purely a dispersion phenomenon of two colloids. Virulent paratyphoid B bacilli of rabbit origin are clumped as readily and disappear as rapidly from the blood as the noninvasive typhoid bacilli. The removal of bacteria from the peripheral circulation is decidedly more rapid in immunized than in normal rabbits and guinea-pigs.

The leukopenia of the peripheral blood following the injection of bacilli is the result of an uneven distribution of the leukocytes which are chemotactically attracted by the bacillary masses collecting in the viscera.

Histologic evidence is presented which indicates that the gallbladders of about one third of the rabbits injected with large doses of typhoid bacilli receive the infection through the terminal capillaries of the mucosa. Preparations made from gallbladders removed on the 24th to 72nd hour after the injection show necroses and diphtheritic inflammatory areas in the villi of the mucosa. The transverse route of bile infection through the wall occurs also after ligation of the cystic duct.

Direct gallbladder injections produce an infection of the wall along the lymphatic system of the mucosa, submucosa and subserosa. The epithelium remains intact and focal necroses of the villi are not observed. In case the typhoid bacilli reach the gallbladder by the hemato-hepatogenous route only, they multiply in the cystic bile, which is suitable for their development. The histologic changes produced in the wall are identical with those provoked by direct inoculation.

Serial sections of the fundus portion of the gallbladder wall removed on the 3rd to 5th day following the infection may indicate on histologic examination the route responsible for the gallbladder lesion, whether descending or transverse.

Rabbit typhoid carriers may be classified into temporary or convalescent and chronic carriers. Thirty to 40% of the intravenously inoculated animals recover from their infection in the first month after the injection. About 10 to 15% may retain typical bacilli in the gallbladder for 6 months to one year, occasionally even longer. The persistence of the micro-organisms depends on the degree of inflammation provoked in the gallbladder. In case the bacillary invasion of the bile is innocuous to the tissues or produces only slight catarrhal cholecystitis, a temporary infection will be noted. It is not unlikely that such a state is regularly the result in descending hemato-hepatogenous infections.

Chronic carriers result probably from embolic, capillary invasion of the wall, with subsequent transverse infection of the bile. Persistence of the bacteria is favored by the formation of biliary calculi (60 to 80% of the cases), by the extension of the inflammatory process to the cystic ducts, and by a severe cholecystitis leading to a loss of contractibility of the wall, followed by a state of empyema. The biliary calculi consist usually of bilirubin-calcium material. The nucleus of this may be an unabsorbed blood clot. Cholesterol stones have not been found. Chronic gallbladder carriers frequently harbor typhoid bacilli in the liver, bone-marrow, lungs and intestines. In exceptional instances isolated foci of infection may be found in the bone-marrow and kidneys. The elimination of typhoid bacilli in the feces of rabbits is, however, practically always associated with gallbladder or bile passage infections. It remains, therefore, to be proved that a close liver focus or any distant focus, provided the gallbladder infection is absent, is in a position to infect the intestinal tract through the bile, to which the organisms are supposed to have been carried by the blood or lymph stream. This contention is sound, notwithstanding the fact that in a few instances it has been demonstrated experimentally that in certain rabbits with infected gallbladders and extrahepatic biliary passages, cholecystectomy does not prevent the elimination of typhoid bacilli in the stools.

Intrasplenic injections of typhoid bacilli produce regularly persistent gallbladder infection. The conception of the human carrier state advanced by Webb-Johnson is discussed.

The insertion of sterile gallstones into the gallbladder of rabbits leads to a secondary infection of this viscus by indifferent streptococci. Such gallbladders are predisposed to a localization of the typhoid bacilli introduced by way of the blood streams.

The typhoid bacillus reaches the gallbladder in human cases of typhoid fever regularly after the liver has been disabled by the poisonous product of the bacteria, and its presence therein is evidenced by positive cultures or by a mild or moderately severe catarrhal cholecystitis. According to the findings of numerous pathologists, the lesions present in the liver and gallbladder are certainly not as severe as is frequently assumed by bacteriologists and epidemiologists. Cholecystitis is a far more frequent complication during typhoid fever than is generally supposed, but purulent necrotizing inflammatory processes are not common, and the formation of bacillary foci in the gallbladder wall supposedly of hematogenous origin is exceptionally rare. Application of the conclusion of J. Koch based on an exceedingly severe and unique form of typhoid cholecystitis is by no means justified.

XX

THE REACTION AND PHYSIOLOGY OF THE HEPATIC DUCT AND CYSTIC BILE OF VARIOUS LABORATORY ANIMALS

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. VI

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In the preceding papers we have shown that typhoid bacilli are frequently discharged in the bile of practically every animal that has been inoculated intravenously with large doses of bacteria of the typhoid-paratyphoid group. Accepting the statements and interpretations of others, it became quite evident that the biliary secretions were under suspicion as important factors in the development of carriers.

Since Nichols issued his studies on the supposedly antiseptic effect of rabbit bile, we were compelled to verify the published facts and to correlate the findings of others with our own. In this connection we studied primarily the antiseptic action of animal and human bile. As Nichols [1] considered the latter to be largely due to its alkalinity, we measured the H-ion concentration of biles derived from a large number of rabbits, guinea-pigs, dogs, rats, cats, etc. We found rather constant difference in the reaction between the bile derived from the liver by a common duct fistula and the one obtained from the gallbladder. We noted that the former became more alkaline on standing outside of the viscus, a final H-ion concentration above P_H 9.0 being frequently reached. Most of our determinations were made on animals that had been kept on controlled diets, and the bile specimens were obtained by temporary common ducts fistulas. In the course of the collection of these samples a number of physiologic data which are rarely mentioned in the literature were recorded. They contribute important information to the physiology of biliary secretions and to the function of the gallbladder, and are therefore included in this paper.

No exact data as to the H-ion concentration of the hepatic duct and gallbladder bile of rabbits, guinea-pigs, rats, monkeys and man were available. Preliminary tests were made by the titration method, but we were convinced that the titrable acidity or alkalinity did not repre-

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[1] J. Exper. Med., 1916, 24, p. 497.

sent the true H-ion concentration of the bile specimen. In measuring the H-ion concentration it was also recognized that special precaution had to be observed in order that the escape of CO_2 did not decrease measurably the H-ion exponent. As stated in the introduction, we have also compared, where possible, the reaction and physiology of gallbladder bile with the hepatic duct bile. In a limited number of experiments we tested the influence of diet on biles, the biles from carrier animals, and followed the changes of bile samples, which had been neutralized in vitro.

METHODS

The hepatic duct bile samples were collected from temporary common duct fistulas. The technical procedures employed have been detailed in the preceding paper.

Cystic bile was procured at necropsies or by laparotomies, a capillary Pasteur pipet being inserted through the seared gallbladder wall and the contents aspirated. In normal healthy animals invariably sterile bile was present. Our observations fail to support the claims of Ehret and Stolz,[2] who recorded that 11 of 17 guinea-pigs' bile tested were contaminated with various organisms. Gallbladder bile of large, domesticated animals was obtained from the slaughter house. The isolated cystic duct was tied and the whole bladder wrapped in gauze soaked with bichloride solution. In the laboratory the bile was aspirated by means of a Pasteur pipet, which was introduced through a cauterized area of the bladder wall. In contrast to Ehret and Stolz, Létienne [3] and others, the samples obtained in this manner were sterile in 90% of the instances.

In studying the reaction of the bile specimens we had access to two methods: the titration method and the colorimetric method. The titration method was handled by us thus: One part of bile diluted in 9 parts of freshly boiled and cooled neutral distilled water mixed with phenolphthalein (0.1 c c of a 2% solution in 50% alcohol to 10 c c of diluted bile solution) was titrated with the aid of a micro-burette (0.1 c c pipet) with a decinormal solution of a sodium hydroxide or hydrochloric acid as the case may be. The end-point arbitrarily chosen by us was the first perceptible change in color recorded during the first minute. The solutions of hepatic duct bile were always clear, and a slight change was readily noted in contrast with the control. Heavily pigmented or turbid cystic biles of man, monkey, dog, etc., were more difficult to titrate. The first discernible change in color covers a broader range and consequently may easily increase the experimental error. Individual differences of interpretation were eliminated by the same person always making the readings.

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[2] Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 350; 7, p. 372.

[3] Arch. de méd. expér. et d'anat. path., 1891, 1, p. 761.

We have deviated from the methods of Jolles [4] and others in slightly varying the amount of indicator used rather than the diluent. For example, we have uniformly used 0.1 c c of phenolphthalein solution for amounts of 0.1 c c to 1 c c of bile. And again in dark and turbid biles we have found it simpler and more expeditious to double or even to quadruple the quantity of indicator rather than to increase the amount of diluent. The comparatively small amounts of bile aspirated from the gallbladder of guinea-pigs and rabbits that was to be titrated before cultivating forced us to titrate only 0.1 c c lots of bile, in which instances the reaction was determined with N/50 solutions of alkali and acid. For measuring the alkalinity of bile samples sensitive azolitmin indicator solution [5] was used in the same manner. However, for the sake of economy in titrations an approximate range of the reaction of biles was first obtained by indicator papers or solutions.

For reasons to be discussed, the colorimetric method for the H-ion determination was adapted for the study of the reaction of bile samples. It is generally accepted that only the accurate determination of the actual hydroxyl-ions should be used to express the reaction of mediums or biologic fluids. Before the publication of Clark and Lubs [6] dealing with a dependable procedure had appeared, and a set of brilliant indicators had been found, we followed the outline described by Hurwitz, Meyer and Ostenberg.[7] It is quite natural that the method of Clark and Lubs, which covers a broader range of reaction, should have replaced the obsolete procedure adopted at the beginning. This in part will explain why some of our results dealing with P_H^+ determinations are expressed as >8.6 and >9.2.

The reaction of the bile is progressively changing and therefore the electrometric method, being time-consuming, would favor erroneous results. However, for old biles exposed to various factors for several days, the electrometric method should be used more frequently. Unfortunately, we were not in possession of an equipment to make such readings when necessary, but through the courtesy of Dr. Schmidt, several samples were checked electrometrically, and the P_{H^+} readings were found to correspond closely with those obtained by the colorimetric method. For biologic work, minor differences of one or two degrees are of considerably less importance than in accurate chemical work. We have recently tested simultaneously a number of old hepatic duct bile samples of the rabbit by the electrometric and colorimetric method and have convinced ourselves that the readings made by the latter are absolutely reliable.

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[4] Arch. f. d. ges. Physiol., 1894, 67, p. 1.

[5] Sörensen: Ztschr. f. phys. Chemie, 1909, 64, p. 120.

[6] J. Bacteriol., 1917, 2, pp. 1, 109 and 191.

[7] Johns Hopkins Hosp. Bull., 1917, 2, p. 2.

As already suggested in the introduction, it was our purpose to determine the reaction of biles, hepatic duct or cystic, in vitro with the least exposure to disturbing factors. It is therefore a great advantage to note from the paper of Clark and Lubs[8] that the "dilution method" is with safety applicable to the H-ion determination of turbid fluids. They established the legitimate dilution as 1:5, but they also mentioned that highly colored solutions may be diluted to a point where they may be used in the comparator. Furthermore, they showed[9] that the 1:10 dilution is reliable for approximate determinations upon a wide variety of solutions highly colored and turbid (p. 193). We have found that a dilution of 1:10 influences the final P_{H^+} readings slightly. It is possible to check this conclusion on lightly colored biles (hepatic duct of guinea-pigs, dogs, etc.) in an undiluted state. A sufficiently large series of comparative tests with highly colored biles has convinced us that a dilution of 1:10 shows approximately the same P_{H^+} values as the one of 1:5 and has also been proved by electrometric determinations.

It has been emphasized that small amounts of bile are in this study the only available material. The dilution method is the only feasible procedure by which we were able to obtain comparable data on the behavior of hepatic duct and cystic bile of one and the same animal. Therefore we have adopted for colorimetric determinations of all biles a 1:10 dilution (0.2 c c bile in 1.8 c c freshly boiled and cooled distilled water mixed with 0.2 c c of indicator). Invariably the P_{H^+} determinations were made with two or more indicators, which cover our ranges, namely: brom thymol blue, phenol red, cresol red and thymol blue. It is always advisable to use at least two indicators overlapping the probable range of the H-ion concentration of the bile. Shades of color which develop under varying CO_2 tensions and which make comparison

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[8] Footnote 6, p. 129.

[9] Footnoe 6, p. 191

TABLE 1

Titrable Acidity and Alkalinity of Varvious Bile Samples

Animal	Diet	Method of Obtaining Bile	Gallbladder Bile					Hepatic Duct Bile				
			Number of Biles Tested	Titrable Acidity Phenolphthalein	Degree of Acidity	Titrable Alkalinity Phenolphthalein	Degree of Alkalinity	Number of Biles Tested	Titrable Acidity	Degree of Acidity	Titrable Alkalinity	Degree of Alkalinity
Rabbit	Mixed	Chloroformed	15	0.8 (0.1-2.7)	0.032	0	0	7	0.13 (4)* (0.1-0.2)	0.52	0.18 (5) (0.1-0.2)	0.064
	Immunized	Or laparotomy						7	0.14 (5) (0.01-0.2)	.....	0.18 (3) (0.1-0.3)	0.064
	Immunized	After death	6	3.5 (2.0-6.4)	1.40	0	0	....	....	.....	....	.....
	Immunized carriers	Laparotomy	18	0.2 (3) (0.1-0.4)	0.08	0.39 (amphotheric —1.1) (15)	0.1404	....	....	.....	....	.....
	Immunized carriers	After death	3	1.5 (0.5-4.0)	0.60	0	0	....	....	.....	....	.....
	Oats	Laparotomy	3	0.46 (0.4-0.6)	0.184	0	0	7	0.06 (2)	0.024	0.15 (5)	0.054
	Wheat	Laparotomy	2	1.12	0.448	0	0	2	0.06 (2)	0.024	0.05 (1)	0.018
	Cabbage	Laparotomy	6	0.61	0.244	0	0	9	0.01 (5)	0.004	0.29 (7)	0.104
	Carrots	Laparotomy	2	0.65	0.260	0	0	2	Amphotheric (1)	.....	0.05 (1)	0.018
	Cholestearolemia	Laparotomy	1	0.2	0.08	0	0	3	0	0	0.13 (3)	0.046
	Alkalosis	Laparotomy										
	Sodium bicarbonate	Laparotomy	3	0.57	0.228	0	0	4	0.3 (1)	0.12	0.16 (3)	0.057
	Acidosis with HCl	Laparotomy	3	0.77 (0.1-2.5)	0.308	0	0	4	0.27 (3)	0.18	0.2 (1)	0.072
	Starvation	Laparotomy	2	1.32	0.528	0	0	2	0.15 (1)	0.06	0.2 (1)	0.072
Guinea-pigs..	Mixed	Laparotomy	47	0	0	0.43 (0.1-1.8)	0.1548	6	0	0	0.55 (6)	0.198
Rats.........	Mixed	Laparotomy	....	....	.....	....	.....	2	0.1 (1)	0.04	0.3 (1)	0.108
Dog..........	Mixed	Laparotomy and after death	33	1.15	0.460	....	.....	5	0.15-0.2	0.06	....	.....
Cat..........	Mixed	Laparotomy	2	1.6	0.64	....	.....	2	0.2	0.08	....	...
Monkey......	....	Laparotomy and after death	7	1.6	0.64	....	.....	1	0.75-1.0	0.3	....	...
Oxen.........	December	2 hours after slaughtering	8	0.55	0.220	....	.....	....	....	.....	....	..
Sheep........	December	2 hours after slaughtering	9	1.9	0.76	....	.....	....	....	.....	....	...
Goats........	December	2 hours after slaughtering	2	0.75	0.30	....	....	1	0.35	0.14	....	.....
Pigs.........	December	2 hours after slaughtering	10	1.05	0.42	....	.....	....	....	.....	....	.....
Man..........	Biliary fistula	Immediately	....	.. ..	.....	....	.....	1	0.1-0.2	0.04-0.08	....	.....
	Sodium bicarbonate and Carlsbader salt	....	....	....	.. ..	....	.....	...	2.2 to lacmoid	.....	....	.....
	Light diet	Cholecystectomy	2	0.8 (1.0-0.6)	0.32	....	.....	....	....	.....	....	.....

* Numbers in brackets refer to the number of animals tested.

difficult with one indicator, frequently can be matched by another, for example, thymol blue is superior to cresol red.

Pyrex glassware was used in all titration procedures. At the beginning of our studies the bile specimens were collected in open 15 c c graduated centrifuge tubes and when filled a colorimetric determination was immediately made. In order to avoid exposure of the bile to the factors changing the reaction, we resorted to the aspiration of the flowing hepatic duct bile by means of a hypodermic "Record" or any tightly fitting syringe. In the course of our inquiry, we finally developed a procedure that accomplishes a similar result in a more convenient manner, namely, the bile is collected directly under a 2 to 3 cm. layer of paraffin oil.

Cystic biles were removed by means of a hypodermic syringe or a Pasteur pipet and transferred to stoppered vials. They were either immediately titrated and tested or placed under oil for a short time.

To avoid a misunderstanding or repetition, we desire to specify that bile specimens standing in test tubes covered with or without oil for from 6 to 10 hours at room temperature or in the ice-chest and stoppered loosely by cotton or cork are considered "old biles."

RESULTS WITH RABBIT BILE

We examined 64 gallbladder and 55 hepatic duct biles of rabbits by the titration and by the colorimetric method. More than 100 gallbladder biles were tested by the colorimetric method only. The results dealing with the titrable acidity and alkalinity are shown in table 1. With the exception of the samples of cystic bile derived from "carriers," which will be discussed separately, all samples were definitely acid to phenolphthalein. The degree of acidity varied from 0.032 to 1.40 mg. of NaOH necessary to neutralize 1 gm. of bile. Rabbits fed on a mixed hay, oats and cabbage diet showed gallbladder bile of lowest acidity; those kept on wheat or starved, or fed with HCl, produced cystic biles that were distinctly acid. The most pronounced acid reaction was noted for gallbladder biles of rabbits which were not necropsied for at least from 5 to 10 hours.

The reaction of the hepatic duct bile of the same rabbits was either amphoteric, very slightly acid, or of various gradations from slightly to decidedly alkaline. About one-half of the specimens immediately titrated were alkaline to phenolphthalein, in contrast to the constant acid reaction of the gallbladder biles. The base-forming diets, particularly cabbage, produced hepatic duct biles, which were distinctly alkaline to phenolphthalein; 1 gm. of bile required 0.104 mg. of HCl for neutralization, using azolitmin as an indicator. Intravenous injections of $NaCO_3$, or feeding of the same substance in combination with base forming diet, gave common duct bile samples which differed little from those obtained from rabbits kept on mixed feeds.

The phenomenon of gradual increase of the alkalinity on standing was also noted when using the titration method. For example, a bile specimen giving a titrable acidity of +0.25 may on standing become amphoteric to phenolphthalein in 24 hours. It is evident that this reaction of the hepatic duct bile is different from that of the gallbladder. As a rule the titrable reaction of the former is more variable and more inclined to the acid side. All hepatic bile specimens were distinctly alkaline to litmus, 23 (or 58%) of 40 gallbladder biles tinged red litmus paper blue, 7 (or 17.7%) were amphoteric and 10 (or 25%) merely changed it to yellow.

TABLE 2

H-Ion Concentration of Gallbladder Bile of Animals Fed on Various Diets

	Number of Animals	P_H^+	Average P_H^+
Mixed diet	15	6.6-7.2	6.7
Mixed diet and fasting	10	5.8-6.8	6.4
Acid forming diet (oats)	5	6.4-7.4	7.0
Experimental acidosis	3	6.4-6.8	6.6
Base forming diets	10	7.0-8.2	7.7
Experimental alkalosis	3	6.8-8.4	7.6

These differences became more apparent when we determined the H-ion concentration of the individual bile samples. Immedately after withdrawal from the gallbladder the bile showed an H-ion concentration expressed in a P_H^+ ranging from 5.7-7.4. The average reaction varied between P_H^+ 6.7-7.0, that is to say, the cystic bile of the rabbit was either acid or neutral or slightly alkaline. It may, however, become strongly alkaline on standing or give an initial P_H^+ reading above 7.2 when the secretion has reached the gallbladder shortly before its removal.[10] Only in exceptional instances did we notice after 24 hours' standing a reaction of less than P_H^+ 8.6. The factors responsible for this alteration will be analyzed. This variability of the reaction of the gallbladder bile is apparently existent in different individuals, and was in our experience closely connected with the general health and the diet of the animals. The figures in table 2 will illustrate this statement.

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[10] The P_H^+ readings of such specimens are recorded in the tables in brackets.

We were particularly interested in the determination of the factors influencing the H-ion concentration of the gallbladder bile and conducted a fairly large series of determinations. The figures noted must be considered strictly as averages; it is difficult to state with absolute certainty the origin of the fluid withdrawn from this organ. In animals with fistula it is not unlikely that hepatic duct bile has regurgitated into the bladder shortly before the sample has been removed and our determinations are made on liver instead of on bladder bile. For practical purposes, it is clear that mixed and acid-forming diets, including HCl are conducive to gallbladder biles which are slightly acid. This likewise is the case in fasting animals, in which there is apt to be stasis in the gallbladder. The reaction on standing remains either stationary or increases slowly. On the other hand, base-forming diets, such as cabbage and carrots and sodium bicarbonate feeding, produce alkaline cystic bile samples, which show a rapidly decreasing H-ion concentration on standing.

Hepatic duct bile, which is withdrawn from the collecting rubber tubing and immediately titrated, is always alkaline. Even when exposure to the air is reduced to a minimum, the P_H^+ reading never goes below P_H^+ 7.5, and in the majority of instances it is between P_H^+ 8.2-8.8. It will be noted from table 2 that the change in H-ion concentration is very rapid and that a bile sample examined 2 hours or more after removal from the body may give a P_H^+ reading from 8.4 to $>$8.6. This change is absolutely constant and is not markedly influenced by the general health of the animal or its diet. We shall discuss our experiments, which were conducted in an endeavor to produce such differences, after we have considered the factors responsible for the progressive changes on standing.

MECHANISM OF THE CHANGES IN H-ION CONCENTRATION OCCURRING IN FISTULA BILE ON STANDING

That changes occur in the reaction of biologic fluids on standing is well known, but to our knowledge particular attention has not been called to this phenomenon in connection with the titration of bile samples. Recent controversies dealing with the H-ion concentration of cerebrospinal fluids, and the careful studies of Levinson [11] suggested that the changes occurring in the hepatic bile on standing resulted from similar causes. A priori we could readily eliminate the conception that the change was due to alkalies given off by glassware. Only carefully tested nonsol or pyrex test tubes were employed in our colorimetric determination. In analogy with the findings of other workers on biologic fluids, it suggested itself that the changes taking place on standing were either the result of loss of CO_2 or the formation or absorption of alkaline substances like ammonia.

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[11] J. Infect. Dis., 1917, 21, p. 556; Cerebrospinal Fluid, 1919, p. 96.

We tested these conceptions by the following experiments: hepatic duct bile collected by aspiration or under paraffin oil in a test tube was divided into 6 portions of from 5 to 10 c c. The reaction of these samples at the beginning of the experiments was P_H^+ 7.8-8.0. Sample 1 was covered with oil and tightly sealed with a rubber stopper; sample 2 was similarly closed, but without oil. Samples 3 and 4 were stoppered, leaving a small air space between the bile and the cork or rubber stopper. Sample 5 was placed in a test tube loosely closed by a cotton plug. Sample 6 was exposed in an open test tube in a desiccator to CO_2 and ammonia-free air. All samples were tested after 10 minutes, 1, 2 and 12 hours. The results of some of these experiments, which were repeated several times, are graphically summarized in chart I.

It may be concluded from these experiments (chart 1) that the H-ion concentration of hepatic duct bile decreases rapidly when the specimens are exposed to alkalies or to open air. The escape of CO_2 can be prevented or reduced by stratification with paraffin oil and tight sealing of the test tube with a cork or rubber stopper. The latter experiment also demonstrates that the decrease in the H-ion concentration is not the result of ammonia production in the fluid. In tubes tightly corked with air bubbles excluded, the bile either retained its original P_H^+ for 12 to 24 hours or was only slightly above it (curves 1 and 2). On the other hand, exposure to NaOH or vacuum produced a rapid decrease of the H-ion concentration showing that accelerated loss of CO_2 is responsible for the increase of the P_H^+.

In a parallel set of experiments, hepatic duct bile collected in an open tube was distributed in lots of 5 c c each in test tubes, which were loosely plugged with cotton. The beginning H-ion concentration of the bile was P_H^+ 8.2 (+0.35). Sample 1 was heated in an open water bath at 56 C. for 30 minutes in order to dispel quickly the CO_2; samples 2 and 3, both not heated, one stratified with paraffin oil and the other exposed to air, were placed with sample 1 in an open water bath at 37 C. Sample 4 was exposed in a chamber with free ammonia; sample 5 in an ammonia-free NaOH-desiccator, and sample 6 in a tube in which the air had been displaced by oxygen. These specimens were incubated at 37 C. P_H^+ determinations were made on all specimens at 1, 2, 24 and 48-hour intervals (see chart 6, second paper).

It was noted that there is little difference in the H-ion concentration of hepatic duct bile when heated at 56 C. for 30 minutes or exposed to air or to oxygen or to an ammonia-free atmosphere in a closed desiccator. However, the specimen exposed to ammonia vapors shows that bile is capable of absorbing it readily. The H-ion concentration was materially lowered. It is suggestive that while the bile may not readily form ammonia-like substances on standing, it may absorb substances like ammonia from the environment This may in a measure explain the variability of the reaction of certain bile specimens when exposed to air or kept in a desiccator. It was frequently noted that one and the same specimen may show a low H-ion concentration (lowest electrometric reading on the 15th day P_H^+ 9.214) when exposed to air, while the reaction of the secretion kept in a desiccator was not lowered in the same degree. In the first few days the desiccator tubes may, as a rule, give a slightly higher P_H^+ reading than the air tube. However, this observation was frequently reversed and the air tube registered a lower H-ion concentration than the desiccator tube. We are under the impression that aside from the escape of the CO_2 another factor may influence the H-ion concentration. The fluctuating ammonia content of the laboratory atmosphere and the varying absorbability of this chemical by the bile may be responsible for the irregular P_H^+ readings. Stratification with oil prevents the absorption of ammonia, but does not entirely inhibit the gradual escape of CO_2. Moreover the P_H^+ increase cannot be the result of an oxidation process. The H-ion concentration of the bile in the oxygen tube was not materially increased in contrast to the one kept under oil. However, what the actual influence of the ammonia absorption by the bile may be, the loss of CO_2, whether rapid or slow, is in our opinion the main factor in the lowering of the H-ion concentration.

Exposure to air therefore produces an hepatic duct bile and, as already stated, also a gallbladder bile which differs materially from the one present in the rabbit body. We are dealing here with a distinct test-tube artefact. Two points deserve, however, a more detailed inquiry in the future, namely: 1. Is the decrease of the H-ion concentration always of the same rapidity in the hepatic duct and the gallbladder bile of individually different rabbits kept under identical conditions? 2. What is the final P_H^+ of such biles when exposed to air for 24 hours? Thus far we possess only a few suggestive observations, namely, experimental alkalosis may produce hepatic duct biles in which the H-ion concentration decreases in a somewhat shorter time than in the secretion obtained from animals kept on acid forming or mixed diets. Aside from the point already mentioned, we call attention to the slight fluctuations in the P_H^+ of fistular bile in the course of a single experiment. In some instances the gradual increase in the H-ion concentration was mainly the result of frequent collections, which were made necessary by the administration of a cholagogue. On the other hand, a slight increase would be readily explained by the gradual CO_2 depletion of the blood and the disturbance of the alkaline reserve balance, which follows the operative procedures and the restriction of bodily movements unavoidable in these experiments. Most of our P_H^+ determinations were made by means of the colorimetric method and represent therefore only relative values. For practical purposes it has been sufficiently proved that the bile of rabbits, irrespective of its origin, changes its reaction when standing exposed to air and that a P_H^+ usually above P_H 8.4-9.0 may be reached in from 12 to 24 hours. The importance of the observation will be appreciated fully in the paper on the antiseptic effect of bile.

ALKALINE RESERVE OF HEPATIC DUCT BILE

We were able to determine the alkaline reserve as bicarbonate on the amount of CO_2 present in hepatic duct bile by the method of Van Slyke and Cullen.[12] It was impossible to obtain sufficient secretion from the gallbladder, which would have enabled us to study the alkaline reserve and the changes in the H-ion concentration simultaneously. Table 3 shows our findings.

TABLE 3
ALKALINE RESERVE IN HEPATIC DUCT BILE

	Blood Plasma, Volume %	Hepatic Bile Duct, Volume %
Rabbit 1418 (mixed diet)	42.4	116.9 on standing
Rabbit 1801 (mixed diet)	50.3	115.2 under oil
		105.7 exposed to air

These data show that the alkaline reserve of hepatic duct bile is more than twice, nearly three times as great, as the one found in the plasma. The average is about 115 volume % of CO_2 found at 0 degrees temperature and 760 barometric pressure. Most of the CO_2 is apparently fixed in form of carbonates, but the additional presence of gaseous CO_2, which escapes on standing, is shown by the lower volume in percentage finding of the specimen of rabbit 1801 when exposed to air. Our plasma bicarbonate figures are lower than those reported by Asada.[13] The differences are probably explained by the fact that we used the heart blood of rabbits which had been operated on, and which had remained in a fixed position on the operation board for at least 6 hours; factors which are known to reduce the alkaline reserve of the blood.

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[12] J. Biol. Chem., 1917, 30, p. 291.

CHANGES OF THE H-ION CONCENTRATION OF THE BILE IN EXPERIMENTAL CONDITIONS

Guided by Nichols's [1] conception that the germicidal property of rabbits' bile in vitro disappears on neutralization with a strong acid (HCl or H_2SO_4) and that therefore the reaction of this secretion governs its destructive influence on bacteria, we conducted tests to confirm the above claims. We furthermore attempted to support his second suggestion, namely, that rabbits in a state of alkalosis produced by a previous injection of sodium bicarbonate are protected against a gallbladder infection. Before one can accept this contention he must show that an experimental acidosis or alkalosis really influences the reaction of the bile. Okada [14] failed to notice a change in the H-ion concentration of fistular bile of dogs fed 200 c c of N/10 HCl solution. On the other hand, we have already recorded the influence of base-forming diets on the biliary secretions of rabbits, a condition which we also attempted to enhance by the use of sodium bicarbonate. We attempted also to solve the next question, namely, is the reverse true? Does the feeding of HCl or a state of experimental acidosis increase the H-ion concentration *in vivo?* The latter procedure gave some suggestive results, but for practical purposes we can probably accomplish in the rabbit the same effect through fasting or the prolonged exclusive feeding of acid-forming diets. A few tests were also made on rabbits which were in a state of hypercholesterolemia.

EXPERIMENTS IN VITRO

Five to 10 c c samples of hepatic duct bile were carefully neutralized with undiluted lactic acid or N/10 HCl. The chemical was added drop by drop, the precipitate which formed under production of gas was redissolved by shaking the tube. A number of tests were also conducted with hepatic bile partially saturated with CO_2. P_{H^+} determinations were made at varying intervals.

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[13] Am. J. Physiol., 1919, 50, p. 1.

[14] J. Physiol., 1915, 49, p. 457; 1915-1916, 50, p. 114.

TABLE 4

The H-Ion Reaction of "Carrier" Biles

Rabbit No.	Diet and Treatment	Agglutination Reaction of Bile	Serologic Findings of Blood Serum		P_H*	Appearance of Bile and Bacteriologic Findings
			Agglutination	Complement Fixation		
941	Mixed diet, laparotomy	1:10	1:1000	0.05 ++	7.2 (2′) 8.4 (60′)	Light greenish, limpid, ∞ B. typhosus
942	Mixed diet, laparotomy	....	>1:20,000	0.0005	9.0 (60′)	Light green, limpid, thick wall, ∞ B. typhosus
943	Mixed diet, killed	....	....	.....	8.0	Light green, limpid, thick wall, ∞ B. typhosus
944	Mixed diet, killed	....	1:6000	.....	8.4	Light green with soft stone. ∞ B. typhosus
946	Mixed diet, killed	1:100	1:1000	0.05	8.2	Clear light green bile, wall thick ∞ B. typhosus
947	Mixed diet	<1:40	1:6000	0.005	8.0	Brownish slimy bile with brownish green débris. wall thick, ∞ B. typhosus
961	Mixed diet, laparotomy	<1.10	1:1000	.....	>8.4 (30′)	Light green, limpid, B. coli
963	Mixed diet, laparotomy	1:10	1:2000	0.05	8.4 (30′)	Light greenish, yellowish slimy sediment, ∞ B. typhosus
671	Mixed diet, laparotomy	1:20	1:1000	.....	>8.4 (30′)	Light greenish bile, sediment, thick wall. B. coli
672	Mixed diet, laparotomy	....	1:400	0.005	8.3 (5′)	Light greenish with adherent crusty sediment, ∞ B. typhosus
676	Mixed diet, laparotomy	1:800	1:1000	.....	8.2	Purulent, colorless, viscid, wall thick
	Recovered at necropsy	1:20 +++	1:500	.....	7.4	Clear yellowish green, sterile
997	Mixed diet, killed	....	1:1000++	.....	7.3	Light green, considerable granular sediment, thick wall, ∞ B. para. B
1035	Mixed diet	....	....	.....	8.0 (10′)	Light greenish, limpid, ∞ B. typhosus
1048	Mixed diet, laparotomy	>1:1000	>1:20,000	<0.005	8.4 (30′) (+0.1)	Light greenish, limpid, very little sediment, wall injected, ∞ B. typhosus
1053	Mixed diet, laparotomy	1:60	1:10,000	0.005	8.4 (30′) (—0.1)	Colorless, sand-like yellow débris wall adhesions, ∞ B. typhosus
1054	Mixed diet, laparotomy	1:20	1:4000	0.01	8.4 (30′)	Clear light green bile in large dilated gallbladder, ∞ B. typhosus
1056	Mixed diet, laparotomy	1:200	1:8000	0.005	8.2 (30′)	Dark green viscid bile, sterile
1057	Mixed diet, laparotomy (coccidiosis, few patches)	1:200	>1:20,000	0.005	8.4 (30′)	Purulent, colorless, slimy bile with considerable sediment, ∞ B. typhosus
1059	Mixed diet, laparotomy	1:20	1:6000+++	0.005	8.4	Light greenish bile with greenish sand-like sediment, wall adherent, abscesses, ∞ B. typhosus
1059b	Mixed diet, laparotomy	<1:10	1:1000	0.005	8.4	Light greenish limpid bile with considerable sand-like sediment, ∞ B. typhosus
1124	Oats, killed	....	1:20,000	.....	7.5	Purulent, slimy with considerable sediment, wall thick, ∞ B. typhosus
1129	Oats, killed	....	....	.....	7.0 (2′)	Purulent, slimy with considerable sediment, wall thick, ∞ B. typhosus
1131	Oats, killed	....	1:10,000	.....	7.5	Greenish, very slight sediment, ∞ B. typhosus
1135	Oats, killed	1:600	....	.....	7.7 (5′)	Purulent slimy, slightly colored, ∞ B. typhosus
1157	Oats and starvation	....	....	.....	7.6	Purulent, colorless, sand-like, greenish sediment, ∞ B. typhosus
1160	Oats, severe infection	1:10–20	1:2000+++	.....	7.5 (5′)	Slimy, colorless, sand-like sediment, 11,800,000 B. typhosus per 1 c c
1163	Oats, Na_2CO_3	<1:10	1:1000	.....	7.8	Light green, limpid, sterile, staphylococcus 100 per c c
27 Rabbits	Average....	1:200 (1:10-1000)	1:400-1:20 000	.....	8.0	

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* If not specifically stated the reactions were determined immediately on collection.

From the data thus collected it became apparent, that neutralization changes only temporarily the H-ion concentration of hepatic duct bile when exposed to the air. In some biles the H-ion concentration decreases progressively in 24 hours until a reaction identical with the original specimen is reached. Preservation of bile under oil delayed this decrease and in most instances the original P_H^+ was not attained. These observations, therefore, indicate that neutralization does not prevent a bile specimen from changing its H-ion concentration. Invariably such fluids exposed to the air reached a reaction which must be considered unfavorable for the growth of B. typhosus and other organisms.

In a few experiments hepatic duct bile of a P_H^+ of >8.8 was partially saturated with CO_2, which increased the H-ion concentration to P_H^+ 6.8-7.0. Exposure to the air in a water bath caused a rapid decrease, and in 24 hours the original P_H^+ was reached. Stratification with oil naturally retarded the return to a low H-ion concentration.

EXPERIMENTS IN VIVO

A small series of rabbits was kept on different diets. Common duct fistulas were made on these animals. The H-ion concentration of the blood was determined by the dialysis method of Levy, Rowntree and Marriot;[15] the the alkaline reserve, R_{PH}, according to the procedure of Marriot; and the reaction of the urine by the colorimetric method. Collections of hepatic duct bile before and after the feeding of varying amounts of HCl or intravenous injection of $NaHCO_3$ were made, and the reactions determined.

In the course of these tests it became evident that final conclusions cannot be drawn from the limited number of experiments. In most instances striking changes in the H-ion concentration of the hepatic bile were not demonstrable. In only two rabbits (692 and 1118), which had been nourished on a mixed oat and hay or cabbage diet respectively for one month, there was recorded a slight decrease in the P_H^+ of the hepatic duct bile during the 2 hour period following the administration of HCl by the stomach tube. In one animal (1118) the R_{PH} of the blood had fallen from 8.6 to 7.8 and the urine also turned acid. In another rabbit (1031 b) the H-ion concentration of the blood decreased following the use of HCl. The R_{PH} remained unaltered, and consequently the influence of the P_H^+ of the hepatic bile was not felt in the two periods following the feeding of HCl, or was so slight that our crude methods failed to record it. We desire also to call attention to the fact that the literature records cases of experimental acidosis in rabbits, in

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[15] Arch. Int. Med., 1915, 16, p. 389.

TABLE 5

ACCORDING TO THE FOLLOWING AUTHORS ONE KILOGRAM OF ANIMAL WEIGHT SECRETES IN 24 HOURS

	Hepatic Duct Bile		Remarks
	Rabbit	Guinea-Pig	
Heidenhain (1883)	136.84 c c	175.84 c c	Gallbladder fistulae (?)
Mann (1918) (4 animals each)	56.84 c c	130.29 c c	Hepatic duct fistulae
Neilson and Meyer (1920)	90.34 c c (17 animals)	154.27 c c (9 animals)	Hepatic duct fistulae
Average	71.34 gm.	153.46 gm.	

TABLE 6

THE RELATION OF BILE SECRETION TO THE BODY AND LIVER WEIGHT OF RABBIT AND GUINEA-PIG

		Rabbit	Guinea-Pig
1. Average weight:	Heidenhain	1525.8	518
	Mann	2158.8	561.8
	Neilson-Meyer	2506.6	899.2
2. Fresh bile per kg. body weight in one hour:			
	Heidenhain	5.070	7.32
	Mann	2.36	5.42
	Neilson-Meyer	3.76	6.42
3. Proportion of liver weight to body weight:			
	Heidenhain	1:33.5	1:27.3
	Mann	1:30.3	1:18.1
	Neilson-Meyer	1:31.1	1:22.09
4 Fresh bile per kg. of liver per 1 hour:			
	Heidenhain	169.3	185.5
	Mann	84.9	130.6
	Neilson-Meyer	122.1	134.5

TABLE 7

THE INFLUENCE OF DIET ON THE RATE OF BILE FLOW PER KILOGRAM WEIGHT FOR RABBITS, GUINEA-PIGS AND RATS

Number of Animals Tested	Diet and Treatment	Average Weight, Gm.	Average Weight of Liver, Gm.	Average Amount of Bile Collected in the First Hour, C c	Average Amount of Bile Collected During 6 Hours, C c	Average Estimated Total of Bile for 24 Hours, C c	Average Capacity of Gallbladder C c	Average Percentage of Bile Secreted in 24 Hours, Which the Gallbladder Will Hold
6 rabbits	Mixed	2,725	90.5	10.09	56.1	218.4 (80.1 per kg.)	3.1	1.4
5 rabbits	Mixed and cholagogue	2,385	73.1	11.0	42.5	156.1	2.0	1.5 (?)
2 rabbits	Fasting	1,990	52.0	5.65	33.7	.....	...	...
4 rabbits	Oats	2,650	76.0	9.75	67.03	279.9 (105.62 per kg.)	...	...
7 rabbits	Cabbage	2,145	75.25	9.69	45.7	182.9 (85.26 per kg.)	1.8	0.96
2 rabbits	Carrots	1,982.5	62.0	10.4	51.75	223.5	2.0	0.7
2 rabbits	Oats and $NaHCO_3$	2,925	112.0	12.0	64.2	256.8	2.8	1.2
9 guinea-pigs	Mixed	899.2	40.7	10.95	....	138.82	1.5	0.96
2 rats	Mixed	298.5	....	0.97	1.3-5.1	23.28	...	...

which the changes of the buffer values of the blood are inconstant, and it is reasonable to suspect that unless the blood alkalies are seriously depleted no appreciable change will be noted in the reaction of the biliary secretions.

It is even more difficult to provoke changes by means of sodium bicarbonate in the H-ion concentration of the hepatic duct bile of rabbits fed with acid-forming diets. The alkali reserve can be readily increased, but in our few experiments this was not followed by any noticeable decrease in the H-ion concentration of the bile. Kurijama and also McClendon, von Meysenberg and Engstrand[17] noted the effect of diet on the alkaline reserve of the blood of rabbits, which is also indicated in our observation by the rather low R_{P_H} of several rabbits. The injection of 1 to 2 gm. of $NaHCO_3$ increased the R_{P_H} slightly, but the alkali depletion (acid diet, narcosis, etc.) was apparently so great that P_H^+ of the urine was only slightly decreased. In one instance it was even increased. And again, in the oat fed rabbits, a marked holding back of alkalies took place. It may therefore be necessary to give more than 2 gm. of $NaHCO_3$, just as is the case in pathologic conditions in order to produce their elimination with the urine or with the bile. The titrable alkalinity also showed no noteworthy increase. It should be recalled that only immediate determinations of the H-ion concentration will give P_H^+ readings which are closely analogous to those of the blood. In a thoroughly alkalinized rabbit (1200) the rapid decrease in the H-ion concentration on standing, and the low final reaction has already been commented on and is undoubtedly the best proof that feeding or injection of alkalies may influence the reaction of bile. How far a moderate alkaline therapy may change the reaction of gallbladder bile cannot be determined satisfactorily on rabbits with common duct fistulas. But we have already recorded in table 2 that 3 rabbits in the state of experimental alkalosis had gallbladder biles with an average P_H^+ reading of 7.6. A similar H-ion concentration can possibly be obtained more readily and with less danger in feeding a base-forming diet, like cabbage and carrots. We will have occasion to consider this point repeatedly in other papers of this series. Judging from a few controlled experiments, a state of acidosis develops during starvation which influences very little the reaction of the hepatic duct bile. This corresponds with the observations of

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[16] J. Biol. Chem., 1918, 33, p. 215.

[17] Ibid., 1919, 38, p. 539.

Asada.[13] The cystic bile is, however, always distinctly acid. A reduction of the plasma bicarbonate concentration is in our experience, so far as the rabbit is concerned, followed by a high H-ion concentration of the cystic bile.[18] The hepatic duct bile remains unchanged. The factors which are responsible for the difference can be determined only by further extensive experimentation.

Experimental cholesterolemia has no influence on the reaction of the hepatic duct bile. The low P_H^+ of the cystic bile of several rabbits is probably the result of fasting or slight under-nutrition. The majority of the animals took the lanolin or brain-carrot mush rather reluctantly, refusing it frequently for several days. The blood of the successfully cholesterolized rabbits contained over 10 times the amount of cholesterol ordinarily found (Bloor [19] — 42 mg. per 100 c c). Hepatic duct bile never contained cholesterol in amounts exceeding 100 mg. per 100 c c of bile.

THE RESULTS WITH GUINEA-PIG BILE

In the course of our experiments we tested 47 gallbladder and 10 hepatic duct bile samples of healthy guinea-pigs kept on a liberal diet of hay, oat and greens. In accordance with the findings of Nichols, we found this secretion to be alkaline to phenolphthalein; the titrable alkalinity varied from 0.43 to 1.8 or 0.154 to 0.720 mg. of HCl per 1 gm. of bile. The P_H^+ was always above 7.0 and H-ion concentration decreased rapidly in a similar manner, as determined for rabbit bile on standing exposed to air. It was practically impossible to collect the hepatic duct bile without exposure to the air. The 2 successful collections gave readings of P_H^+ 7.7 and 7.8. When compared with the cystic bile of the same animals, we found that the P_H^+ of the latter was only 7.2 (normal—P_H^+ 7.55). These figures suggest, but do not prove, that hepatic duct bile is slightly more alkaline than cystic bile. In the majority of instances, however, the cystic bile differed from the hepatic duct bile neither in its reaction, color nor physical consistency. In the animal body the H-ion concentration is probably identical with that of the blood. Guinea-pigs suffocated or moribund with a blood P_H^+ of 7.0-7.2 due to an accumulation of carbon dioxide gave gallbladder specimens which were neutral or slightly alkaline (P_H^+ 7.0-7.1) when tested immediately. The data available illustrate, however, the progressive decrease on standing.

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[18] Hirsch: Jour. Am. Med. Assn., 1920, 75, p. 1204.

[19] J. Biol. Chem., 1916, 24, p. 227.

THE RESULTS WITH DOG, CAT, GOAT, RAT, MONKEY, OX, SHEEP AND PIG BILE

The measurements of the H-ion concentration of the hepatic duct and cystic bile of a series of different animals gave varying results. Two facts were demonstrated: 1. The reaction of the gallbladder bile is always more on the acid side and varies more than the hepatic duct bile. 2. Both secretions change their reaction on standing. The decrease in the concentration is apparently more rapid, and a lower P_H^+ value, namely 8.2-8.4, is reached in dogs (five) fed and injected intravenously with sodium bicarbonate. Our findings corroborate fully the observations of Okada,[14] who states that it was necessary to keep dog bile free from air, otherwise the value of the H-ion concentration is found to be subject to change.

In connection with our problem on the antiseptic effect of bile, it was of particular interest to record the progressive decrease in the H-ion concentration of gallbladder bile of oxen. Some samples of bile collected aseptically from the gallbladder in from 1 to 2 hours after death and stored in cotton stoppered test tubes, reached a H-ion concentration of P_H^+ 8.4-9.0. The entire bacteriologic literature dealing with the antiseptic and the inhibitive effect of ox bile has apparently overlooked this phenomenon. Invariably heated sterilized ox bile collected in San Francisco abattoirs has a reaction which certainly cannot be considered ideal for the growth of organisms of the typhoid-paratyphoid group, and yet no attempts have been made to adjust this important factor. Is it not possible that some of the reports and the various contradictory statements relative to the germicidal effect of ox bile can be ascribed to this unrecognized factor? Is perhaps the reduction of the inhibitory effect of ox bile on B. typhosus by the addition of glycerol (E. E. Ecker [21]) explained by the fact that impure glycerol is slightly acid and could therefore improve an alkaline bile specimen as a culture medium. All of these questions will be answered in the next paper, but it is sufficiently manifest that attention should be called to the alkaline reaction of old ox bile and to the possibility that chemical transformation takes place more readily in such an environment than when the medium is neutral.

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[21] J. Infect. Dis., 1918, 22, p. 95.

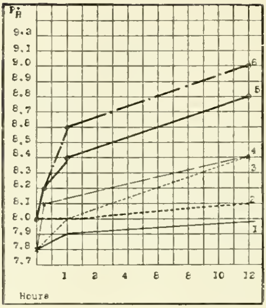

Chart 1. Change in H-ion concentration of hepatic duct bile on standing at room temperature. 1, rabbit 1481—collected under paraffin oil sealed with rubber stopper; 2, rabbit 1481—corked with rubber stopper, a few bubbles at the top; 3, rabbit 1182—partially filled tube, sealed with rubber stopper; 4 rabbit 1182—partially filled tube corked with cork; 5, rabbit 1200—exposed to air, plugged with cotton; 6, rabbit 1200—exposed to CO_2 free air in desiccator.

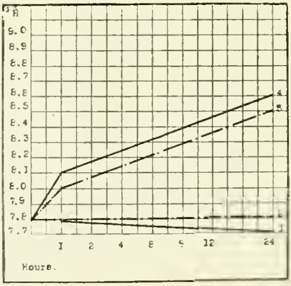

Chart 2. Change in H-ion concentration of gallbladder "carrier" bile on standing at room temperature. 1, rabbit 1625—bile containing 22,500,000 B. typhosus per c c kept under sterile condition in the removed gallbladder; 2, rabbit 1630—bile containing 80,000,000 B. typhosus per c c kept under sterile condition in the removed gallbladder; 3, rabbit 1630—bile in small tube closed with cotton stopper then incubated and found sterile in 4 days; 4, rabbit 1625—bile in small tube closed with cotton stopper, then incubated and found sterile in 15 days after removal.

The only gallbladder bile specimens which remained unchanged on standing or showed after 24 hours an increased H-ion concentration were derived from sheep. In tightly corked tubes the P_H^+ decreased from 7.2-6.9. This indicates that there is not only no loss of CO_2, but there also is probably a formation of lactic acid on standing. Further tests will tell whether this increase in the H-ion concentration is characteristic for gallbladder bile of sheep.

Bile samples obtained by fistula from rats behaved like liver bile and were decidedly alkaline. The gallbladder bile of monkeys was acid or alkaline; in one instance the hepatic duct bile was found alkaline.

In connection with our colorimetric measurements of the H-ion concentration of these bile specimens we conducted a series of titration tests. We can in general confirm the observations of Jolles [22] Chittenden and Albro [23] and others. The cystic bile of oxen, sheep, goats, pigs, cats, dogs and monkeys is acid to phenolphthalein. From 0.22 to 0.76 mg. of KOH or NaOH were required to neutralize the acid salts of 1 gm. of the different specimens. This acidity varied considerably for the individual animal of the species, and it is apparent that the same factors cited for the differences in the H-ion concentration, namely, the general health of the animal and its diet, influence the titrable acidity or alkalinity. To these two factors we desire to add a third, namely, the time period after death elapsing between the collection of the biliary secretion from the gallbladder gradually becomes more acid. Those exposed to the air in test tubes become more alkaline. The data on which these statements are based was obtained in titrating the specimens as fresh as possible, at least within two hours after death, and immediately after their collection from the gallbladder. Adherence to this principle may in part explain the lower acidity noted in our series in comparison with findings reported by Chittenden and Albro [23] and those of Jolles.[22]

The titration method also shows that the hepatic duct bile is different from the gallbladder bile. Invariably the former is very slightly acid to phenolphthalein; the majority of the 1 gm. bile samples required less than 0.1 mg. of NaOH for neutralization. These specimens were uniformly alkaline to litmus.

RESULTS WITH HUMAN BILE

As we found that the reaction of gallbladder bile retained in the rabbit body for some time was subject to change, we considered it unnecessary to study specimens collected from necropsy examinations. Aside from a few samples obtained from gallbladders after cholecystectomy, we were fortunate in having a case of biliary fistula in the wards. The biliary secretion was collected for a number of days, and the reaction of the same was determined by the titration and by the colorimetric method. The patient felt more comfortable, and the liver bile was less mucoid, when sodium bicarbonate (6 gm. daily), Carlsbad salts and magnesium sulfate were freely administered. This alkaline therapy has to be taken into consideration in judging the reaction of the bile, which was always alkaline to litmus; 1 gm. of bile required on the average 0.72 mg. of HCl to neutralize the mono- or di-basic phosphate salts, using lacmoid as an indicator. As a rule, the clear, light greenish fluid was either neutral to phenolphthalein, or 0.04 to 0.08 mg. of NaOH were necessary to neutralize the specimen using the same indicator. The P_H^+ was usually 8.0 and increased on standing to 8.6. Heated specimens always had a P_H^+ above 8.4. The latter procedure was necessary to insure sterility of the biliary fluid, which contained a few paracolon bacilli and B. coli aerogenes. The few gallbladder specimens obtained from cholecystectomy cases were alkaline to litmus. The H-ion concentration was low; the range extending from P_H^+ 7.7-8.6. We have the impression that the specimens were actually liver bile recently discharged into the gallbladder. The bile was light colored, limpid and of low concentration.

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[22] Arch. f. d. ges. Physiol., 1894, 67, p. 1.

[23] Am. J. Physiol., 1898, 1, p. 307.

In general, our findings agree with those already published. Pisenti,[24] Brand,[25] Copeman and Winston [26] found human fistula bile to be neutral or faintly alkaline; Fränkel and Krause,[27] Toida [28] and others state that the majority of human gallbladder biles were neutral, a few alkaline, but never acid to litmus.

THE RESULTS WITH GALLBLADDER BILE OF RABBIT TYPHOID OR PARATYPHOID CARRIERS

In examining the gallbladder bile of "carrier" rabbits, we employed the methods outlined in the previous paragraphs. Since 1917 more than 50 specimens have been carefully tested, but only an unselected number of 27 are tabulated in table 4. We found that "carrier biles" differed in no respect from those obtained by fistulas, the P_H^+ being on the average 7.5 immediately on withdrawal from the laparotomized chloroformed animals. In not one instance did we find a bile specimen in which the P_{H^+} was below 7.0; in most instances the P_{H^+} was 7.3-7.6 (normal P_{H^+} 7.22). On standing these samples usually decreased in a shorter time than normal hepatic duct bile and reached P_{H^+} 8.0 within 1 to 2 minutes and above 8.6 in less than one hour. Beckwith[29] made the same observation. Immediately tested all specimens were alkaline to litmus and were either alkaline (0.1) or neutral or very slightly acid to phenolphthalein. In consistency the biliary secretions were limpid, clear and light greenish. These were at times free from pigment, with a sand-like, yellowish-green granular carbonate sediment (up to ⅓ of the total fluid bulk) and microscopically had a débris which consisted of leukocytes, epithelial cell and masses of bacteria. The samples rich in carbonate débris, as is to be expected, changed their reaction rapidly to a low H-ion concentration. Others were slimy and pus-like in character and retained their acidity for a longer period than the clear limpid fluids; the H-ion concentration either remained stationary or increased slightly. This phenomenon is shown in chart 2, curve 2. A carrier bile rich in exudate behaves, therefore, in a somewhat similar manner to spinal fluids derived from meningococoric meningitis (Levinson[30]). It is not unlikely that the mechanism underlying the slow decrease in the H-ion concentration is the same, namely, lactic acid formation due to a destruction of cells on standing. However, owing to the rather small number of purulent carrier biles studied, we are not prepared to offer this suggestion as a final, conclusive statement.

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[24] Arch. Med. Ital., 1890, 14, p. 13.

[25] Arch. f. Physiol., 1902, 90, p. 491.

[26] J. Physiol., 1889, 10, p. 213.

[27] Ztschr. f. Hyg. u. Infectionskrankh., 1889, 32, p. 97.

[28] Arch. f. klin. Chir., 1913-1914, 103, p. 407.

The H-ion concentration of carrier bile stands apparently in close correlation with the physiologic activities of the mucous membrane of the gallbladder. Thickening of the wall with signs of an inflammatory reaction always revealed a limpid bile which behaved similarly to hepatic duct bile. The gallbladder of a "carrier" animal has apparently lost its concentrating function and therefore fails to change the reaction of its content. On recovery from the carrier state, which leads to sterile bile samples and which is accompanied by an increased viscosity and pigmentation, the H-ion exponent returns to the normal variant P_{H^+} range from 6.8 to 7.6. As determined repeatedly, the mere presence of bacteria in the gallbladder bile does not influence the H-ion concentration. A rabbit, for example, in a fasting condition with a typhoid focus in the liver, which constantly discharges B. typhosus into the bile, but without involvement of the gallbladder wall, may give a P_H^+ reading of 6.4-6.8 plating of the biliary fluid, may demonstrate millions of viable bacteria. It will be our object in the next paper to consider the relationship of the reaction of the standing carrier biles to the progressive sterilization from B. typhosus. Early in our studies we noted that irrespective of the low H-ion concentration, the bacteria present in carrier bile may remain viable for more than 20 days. These observations cast considerable doubt on the conception that the reaction of the bile is the most important factor in the development of a carrier state in rabbits. This drift of thought prompted the diversified studies given above, which in our opinion have shown that in all probability the H-ion concentration of the gallbladder is most suitable for the development of bacteria of the typhoid-paratyphoid group. With the progress of the infection, when changes in the gallbladder wall ensue, the H-ion concentration becomes more or less identical with the one of the blood.

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[29] Thesis, 1920.

[30] Cerebrospinal Fluid, 1919, p. 142.

We were unable to correlate the presence of agglutinins in carrier biles with the reaction of the same specimen. Most of our samples were kept in the ice-chest or at room temperature for at least 24 hours before they were used for serologic tests. Their reaction was constantly above P_H^+ 8.0; low or high agglutination tests were encountered. The H-ion concentration, therefore, cannot be considered the factor which governs the agglutination reactions of carrier biles.

DISCUSSION

The reaction of the bile of laboratory animals is treated only in a few scattered statements of the literature. Unfortunately, these notes are not strictly comparable on account of the variety of methods, which are used for the determination of the reaction. In most instances no distinction is made between the hepatic duct and the cystic bile.

Chittenden and Albro[23] measured the alkalinity of rabbit bile flowing directly from the liver through a fistula and not coming in contact with the gallbladder. They found that it had an alkalinity of 2.5 mg. HCl per gm., using phenolphthalein as an indicator. There was no measurable acidity, and these workers therefore concluded that this bile may contain an alkali as strong as sodium carbonate. Nichols[1] titrated his bile samples using phenolphthalein and lacmoid as indicators and noted a neutral or 3 to 6.0 alkaline reaction in rabbit bile. Aside from Quagliariello,[31] who reported the H-ion concentration of gallbladder bile of the rabbit to vary from P_H^+ 6.4 to 7.9, no records of similar determinations by modern methods are available. Apparently no attention has been paid to the changes occurring on standing, and we are therefore unable to establish definitely from the data collected the normal reaction of the liver and of the gallbladder bile of rabbits. Neither could we find statements made with regard to the diet and the general conditions of the animals, which were used for the determinations of the bile reactions.

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[31] Rendiconti Acc. dei Lincei, 1911, 22, p. 302

Our findings as a whole support the contention of Chittenden and Albro.[23] The fistula bile of rabbits is alkaline to phenolphthalein and this alkalinity is probably due to carbonates, as base-forming diets increase noticeably the average titrable alkalinity. The H-ion concentration of the hepatic duct bile varied between P_H^+ 7.6-7.9; it was never below P_H^+ 7.5. We are fully convinced that if technical difficulties could be overcome, the reaction would be found to correspond with the one determined for the blood, which according to Hasselbalch [32] is P_H^+ 7.33, (at 38 degrees) and for the rabbit P_H^+ 7.65 (Dragstedt [33]). A well balanced equilibrium apparently exists between the CO_2 tension of the blood and the biliary secretions. This explains our difficulties in changing the H-ion concentration of hepatic duct bile by various diets, HCl feeding or intravenous injection of $NaHCO_3$, even when the titrable acidity or alkalinity differed from the normal average. Misleading values of the H-ion concentration can, however, be obtained when the bile samples are exposed to the air for some time; low concentrations are regularly recorded. We found that the P_H^+ ranged from 8.2 to >8.6; readings of over 8.2 being sometimes obtained 10 minutes after removal from the animal body. Our tests have shown that in the first place these changes follow the escape of CO_2; then possibly the absorption of ammonia.

Gallbladder bile of rabbits was found in a large series to be acid to phenolphthalein. Colorimetric determinations, on the other hand, have shown that these biles can be acid, neutral or alkaline. The P_H^+ range extends from 5.7-7.7. The variability of the reaction exists in individual rabbits and seems to be connected with the general health and the diet of the animals. Fasting, disease and acid-forming diets and HCl feeding produce an acid reaction in contrast to the alkali increasing effect of base-forming diets. Also the cystic bile changes on standing, perhaps less rapidly and inconstantly than the hepatic duct bile. The final H-ion concentration of such samples does not decrease below P_{H^+} 8.0-8.2. This is particularly true when the specimens have been retained in the gallbladder for at least 2 to 6 hours. Concentrated, viscid biles are also less subject to changes than the more dilute limpid specimens. It will be clear from this statement that the cystic bile of rabbits never reaches even in vitro a reaction which is destructive to bacteria, for example, B. typhosus. In most instances in vivo the H-ion concentration of the gallbladder bile will be P_{H^+} 6.8-7.0. Schoenholz and Meyer have shown that this reaction in salt-free buffered broth favors the optimum growth of B. typhosus. In the light of all these findings it is not logical to accept Nichols's conclusions based on in vitro experiments that rabbit bile is antiseptic on account of its alkalinity.

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[32] Biochem. Ztschr., 1911, 30, p. 317.

[33] Jour. Infect. Dis., 1920, 27, p. 450.

As far as we have been able to survey the literature, no one has as yet determined the H-ion concentration of rabbit carrier bile. We found that such biles behave like the hepatic duct specimens or biologic fluid rich in leukocytes. On standing they either show decreasing, stationary or increasing H-ion concentrations. In vivo the reaction is probably identical with the one characteristic for the blood of the individual rabbit and most suitable for bacterial growth. In vitro a reaction is sometimes reached which must be considered unfavorable for the bacteria of the typhoid-paratyphoid group. Germicidal properties develop only when other factors have made their occurrence, as will be discussed in detail in the next paper.

Nichols first called attention to the strongly alkaline reaction of the guinea-pig bile. As far as titrable alkalinity is concerned, we can fully support his findings; however, the H-ion concentration is high for freshly measured biles and decreases rapidly on standing. In the guinea-pig body the reaction is identical with the one of the blood.

Our H-ion concentration measurements on dogs, cats, goats, oxen, sheep, pigs, rats, monkeys and human bile correspond with those reported by Okada [14] and Quagliariello.[31] The reaction of the bile from the gallbladder and from the hepatic duct is different. The reaction of the former is more variable and inclines toward the acid side. The general condition and diet of the individual of the same species are in part responsible for this variability. And again, the average titrable acidity or alkalinity varies for different species. With the exception of sheep bile, most of the gallbladder biles exposed to the air show a decrease in the H-ion concentration on standing. The escape of CO_2

is responsible for this change and is particularly striking in ox bile. This fact should be carefully considered in future studies on the inhibitive and antiseptic affect of this secretion.

The hepatic duct biles of dogs, cats and one human case of biliary fistula were slightly alkaline when tested immediately on withdrawal from the body; they also changed on standing. A carefully adjusted equilibrium between the H-ion concentration of the blood and the liver existed apparently also in these species, just as we discussed it for the rabbit and guinea-pig. The liver bile of dogs which are in a state of experimental alkalosis shows a more rapid fall in the H-ion concentration than the hepatic duct bile of the normal.

The numerous experimental bile collections made in the course of our study, supply a number of physiologic data, which have not only a bearing on certain phases of our problem but contribute to the knowledge of the function of the gallbladder and the importance of the biliary secretions in general. These facts are briefly enumerated in the following paragraphs.

RATE OF BILE-FLOW AND THE INFLUENCE OF CHOLAGOGUES

Any one working with animals possessing a biliary fistula is at once struck by the fact that the small herbivorous animals, rabbits and guinea-pigs, secrete considerably more bile in a given time interval than the omnivorous and carnivorous species. When comparing our quantitative findings, recorded in tables 5 and 6, it will be seen that the guinea-pig secretes usually more than twice the amount of bile in 24 hours that the rabbit does. Our figures are based on a considerably larger series of animals than those published by Heidenhain [34] in 1883 and by Mann [35] in 1918. Their estimates are not strictly comparable with our own for these reasons: Mann made the collections from animals while under an anesthetic, which in his opinion probably decreased the secretion. The data presented by Heidenhain were presumably collected from the work of Bidder and Schmidt [36] and were made in a period (report published 1852) when anesthetics were not regularly used in animal experiments. It is our custom to let the animal completely recover from the ether before the rate of bile flow is estimated. We possess, however, sufficient data which show that this operative procedure had but a slight influence on the output of bile per hour. In contrast to Mann, we determined the bile secretion for at least 6 hours and estimated our total daily output from this figure. We have gradually become accustomed to consider the average hourly output as 10 c c for rabbits and guinea-pigs. There are individual differences, and temporary reflex inhibition or dislocation of the cannula may influence the total collection. According to our figures, the daily output of bile represents $\frac{1}{8}$ to $\frac{1}{10}$ of the total body weight of the animal (table 7).

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[34] In Hermann's Handb. d. Physiol., 1883, 5, Pt. 1, pp. 249 and 412.

[35] New Orleans Med. & Surg. Jour., 1918, 71, p. 80.

[36] 1852, p. 191.

The rate of flow is not perfectly uniform. Some animals show a decided decrease in secretion toward the 5th and 6th hour of the experimental period, others a marked increase. These differences are more or less individual, and their cause cannot be easily explained. Improperly secured and restless animals increase by their bodily movements the rate of the bile flow. The diet changes the total 24-hour output. A strict oat diet with a liberal amount of water apparently stimulates bile secretion. The base-forming diet, like hay, cabbage and carrots, produces on the average 80 to 90 c c of bile per 1 kg. weight of rabbit in 24 hours. It is not unlikely that the findings of Bidder and Schmidt [36] are partially explained by the fact that their experimental rabbits were kept on a liberal hay and oat diet. Our observation corroborates the statement made by Abderhalden [37] and suggests that in future determinations of the rate of bile flow proper attention should be paid to the diet of the animals. Unfortunately only two experiments on fasting rabbits are available for comparison. In both rabbits the rate of bile flow was so markedly decreased that even the use of cholagogues failed to increase the hourly output to a noteworthy degree.

As already stated, the reflex inhibition influenced the rate of bile flow in the dog, cat, goat and monkey. Accurate determinations of the amount of bile secreted are therefore impossible.

In the 2 rats successfully operated on and kept under observation for from 12 to 24 hours, the flow of bile was irregular; in one animal the hourly output at the beginning of the experiment was more than 1 c c, in the second it was less than 0.5 c c. The total amount of bile collected in 24 hours represents about one-ninth to one-tenth of the body weight.

In a case of human biliary fistula in which the patient received sodium carbonate and Carlsbad salt, the hourly rate of bile flow fluctuated between 13.2 to 67.4 c c. The total secretion estimated on collections made for 6 hours on 5 successive days averaged 885 c c for 24 hours. The secretion was never continuous and was clearly correlated with the bodily movements. According to figures published by Copeman and Winston[38] (779.6 c c for 24 hours), Pfaff and Balch (88) (525 c c) and others our averages appear rather high. It is not unlikely that the alkaline therapy may in part be responsible for this increased rate of bile flow.

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[37] In Ellenberger and Scheunert: Lehrbuch d. vergleich. Physiol. d Haussäugetiere, 1910, p. 267.

The effect on the bile flow of ox bile feeding and the intravenous injection of sodium taurocholate in rabbits was observed in the course of several experiments. The feeding of from ½ to 1 ounce of ox bile in two experiments failed to increase the rate in the 3 to 4 hours subsequent to its administration. Practically the total amount of bile introduced was found in the stomach at necropsy. Intravenous injection of from 200 to 500 mg. of sodium taurocholate produced, as is indicated in table 3, an immediate but temporary stimulus in the rate of bile flow. The hourly average of 5.6 c c increased in one instance to 13.5 c c and in another from 2.8 c c to 9.3 c c. As a rule, the familiar cholagogue action of the taurocholates was spent within the next 2 to 4 hours, depending on the amount injected.

COLOR OF THE BILE

In the course of our experiments we found that the changes in the color of the bile of different species of animals may serve as a valuable guide in judging the age of a specimen. It appears therefore necessary to give a brief description of the various color changes developing a result of oxidative or reductive processes. Rabbit bile secreted from the common duct is clear and has a light green color, which usually deepens to a smaragadine green color on standing. Exposed to the air the sterile fluid acquires in the course of a week a slightly brownish shade. The green gallbladder bile changes more rapidly to a deep brown color. After 2 days usually the bilirubin is transformed to hydrobilirubin. When protected from oxidizing processes by stratification of the sample with paraffin oil, the color remains unaltered for at least 2 weeks.

The hepatic duct bile of the guinea-pig is a light golden yellow color which on exposure to the air becomes a dull green or even a light brown. Cystic bile usually is of the same color; in some animals the secretion may be entirely colorless. A similar color scale can be noted in rat bile.

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[38] J. Physiol., 18[illegible] [illegible] 2[illegible]

The bile of dogs, cats, monkeys and man secreted from the liver is a light brownish yellow, sometimes with a greenish tinge which changes on standing to a dull brown or golden brown. The gallbladder bile of these species, including pigs, may vary from an olive green to a dull reddish brown color, depending entirely on the age of the specimen.

Ox, goat and sheep bile is olive green when fresh, but changes to a dull brown when exposed to the air. Retained in the gallbladder for more than six hours, a similar change is noticeable. The alterations in the color are usually accompanied by definite changes in the reaction of the fluid. This important fact has already been discussed in detail.

THE FUNCTION OF THE GALLBLADDER AND THE DIFFERENCES BETWEEN HEPATIC AND CYSTIC BILE

In the course of a study of the biliary secretions as a medium for the development of bacteria it becomes apparent that certain differences exist between the bile collected from the hepatic duct and the one procured from the gallbladder. Irrespective of the fact that our knowledge concerning the function of the various biliary secretions and particularly of the gallbladder is meager, a brief review of the essential positive findings, as far as they concern our problems, is herewith attempted.

In the light of the work of Mann,[35] Rost [39] Rous and McMaster [40] and others it must be recognized that the gallbladder has a mechanical and probably a chemical function. The gallbladder influences the flow of bile and acts as a current regulator of the hepatic duct bile. Observations made by Rost,[39] Eisendrath and Dunalvy [41] show that usually all the ducts outside the liver dilate after the removal of the gallbladder. The work of these investigators indicates, that at least in certain species of animals, the gallbladder has a definite function. Okada [14] has demonstrated rhythmic contractions in the gallbladder which increase during the height of digestion and after the administration of acids. Mann [35] noted inactivity of the sphincter of Oddi in animals without a gallbladder. The secretions of the bile in such species is continuous. In animals with active sphincters, a gallbladder is necessary to regulate the bile secretion and discharge and to prevent the fluctuations in the intraduct pressure.

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[39] Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1913, 26, p. 710.

[40] Jour. Exper. Med., 1920, 32, p. 249; Proc. Soc. Exper. Biol. & Med., 1920, 17, p. 159

[41] Surg., Gynec. & Obst., 1918, 26, p. 110.

The mechanism controlling the action of this viscus is, according to Meltzer,[42] under nervous control. Disturbances of the law of contrary innervation, in his opinion lead to pathologic stasis. Fasting, starvation or irregular partaking of food does not supply the necessary peptones, which cause the bladder reflex to discharge the bile. In rabbits we have noted the influence of starvation in producing stasis of bile in the gallbladder.

Our observations also confirmed the recent studies of Mann, which showed that the gallbladder of no species of animal is capable of holding more than from 2 to 5% of the total amount of bile secreted in 24 hours. The function of the gallbladder as a reservoir in the same sense as the urinary bladder is not entirely justified, even if we admit that periodical storage in the gallbladder has probably a certain protective advantage to the intestines or some of its functions.

The bile collected from the gallbladder is more concentrated than that withdrawn from the hepatic duct. Hammersten [43] found from 1.11 to 1.19% solids in the hepatic and from 8 to 10% in the cystic bile. This difference is the result of the concentrating function and said to be due to an absorption of water, but it is now considered to be caused by the addition of material produced by the cells lining the outer biliary passages. In this way mucous material (mucin, phosphoprotein) and some cholesterol are added to the hepatic bile. Their functional significance is not definitely understood: according to some investigators, the mucus alone is added, and it has no other function than that of annointing the surfaces of the biliary channels and intestines. Rost [39] was convinced that the gallbladder bile, which has 10 times more active biliary alkalies and 8 times more solid substances than that of the liver, contains material of greater importance to the digestive tract than the hepatic duct bile. Furthermore, it has been repeatedly noted in rabbits that the contents of a diseased carrier gallbladder are limpid, free from mucus and correspond in every respect with the secretion obtained from the hepatic duct. In such cases the mucous membrane of the gallbladder is practically destroyed by the subacute inflammation provoked by the vegetating B. typhosus.

The excellent studies of Rous and his collaborators definitely indicate that the gallbladder mucous membrane is endowed with absorptive properties, which act with great rapidity. Observations on cholesterolized rabbits by Dewey[44] and ourselves suggest that cholesterol is deposited on the epithelium of the gallbladder as a result of resorption from the bile and is not a product of secretory activity of these cells. In a few experiments primarily conducted for an entirely different purpose, we found that rabbits eliminated a large percentage of the injected cholesterol through the bile. Particularly in the rabbits, which showed anisotropic fat deposits in the gallbladder, the hepatic duct bile contained a rather high percentage of cholesterol. Experiments on cholesterolized rabbits with common duct fistulas therefore supplied valuable information concerning the mooted origin of the biliary cholesterol.

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[42] Am. J. Med. Sc., 1917, 153, p. 469.

[43] Lehrbuch der physiologischen Chemie, 1914, p. 390.

Various other aspects of the function of the gallbladder, as a reservoir for pathogenic micro-organisms thriving either in its contents or its wall, will be presented in the course of our findings on experimental gallbladder carriers.

SUMMARY

The hepatic duct bile of rabbits is always alkaline to litmus and frequently also the phenolphthalein, the P_H^+ varies between 7.4 to 7.7, if examined immediately on withdrawal from the body. The H-ion concentration of this bile decreases steadily on exposure to air on standing and may reach a final P_H^+ of 9.2. If the bile is collected under paraffin oil or put in tightly corked tubes, this change does not take place as readily. The decrease is probably the result of an escape of CO_2 and the absorption of ammonia. The reaction of the bile from the gallbladder is variable; it may be acid, neutral or alkaline, but it is always acid to phenolphthalein. The H-ion concentration of the cystic bile is influenced by the health and the diet of the individual animal and may have a P_H^+ from 6.4 to 7.7, average P_H^+ 7.22. On standing also a decrease in the H-ion concentration takes place which is more rapid for animals kept on base-forming diets. Fasting and acid forming diets produce cystic biles of a high H-ion concentration. Feeding of HCl or injection of $NaHCO_3$ may influence the reaction of the gallbladder bile. Alkaline hepatic duct bile, when neutralized with acid and exposed to the air, regains on standing its original low H-ion concentration.

The hepatic duct and gallbladder biles of guinea-pigs differ little in reaction. The bile is strongly alkaline to litmus and moderately so to phenolphthalein. Fresh bile has a P_H^+ of about 7.5 which changes rapidly, on standing, to a low H-ion concentration.

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[44] Arch. Int. Med., 1916, 17, p. 757.

The reaction of the bile from the hepatic duct and from the gallbladder is different in the dog, cat, goat and monkey. The gallbladder bile reaction is always more variable and inclines toward the acid side. The cystic bile of oxen, sheep, and pigs is faintly alkaline to litmus, the P_H^+ ranges between 7.0 and 7.5 on fresh specimens but on heating or exposure to the air it changes steadily in some instances to a P_H^+ above 8.4.

Human fistula bile of one case was faintly alkaline to litmus and faintly acid to phenolphthalein. The P_H^+ 8.0 increased on standing to P_H^+ 8.6. Gallbladder bile from cholecystectomy cases showed a P_H^+ range from 7.7 to 8.6.

Biles derived from typhoid or paratyphoid or streptococcic infected gallbladders of rabbits are alkaline to litmus, and about 50% of them are also alkaline to phenolphthalein. The P_H^+ varies between 7.3 and 7.6 and frequently decreases rather rapidly on standing. They behave in general like hepatic duct bile specimens. Purulent gallbladder specimens may show, on standing, a stationary or even an increasing H-ion concentration, probably due to the formation of lactic acid provoked by the disintegration of cellular material.

The average hourly rate of the bile flow of rabbits is approximately 10 c c and in 24 hours about $\frac{1}{8}$ to $\frac{1}{10}$ of the body weight. One kg. of rabbit secretes 3.76 gm. and 1 kg. of guinea-pig 6.42 gm. of bile per hour. Intravenous injections of sodium taurocholate produce a temporary cholagogue effect in rabbits, dogs and cats. Particularly in the latter species, the familiar cholagogue effect of sodium taurocholate is of great assistance in overcoming reflex inhibition, which follows operative procedures employed in the production of temporary common duct fistulas.

The mechanical and chemical function of the gallbladder is discussed.

THE BACTERIOSTATIC AND GERMICIDAL PROPERTIES OF BILE

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. VII

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Synopsis

Introduction.
Brief review of the literature.
Methods.
The qualitative germicidal action of hepatic duct and gallbladder bile of rabbits, guinea-pigs and dogs.
Notes on the basic qualitative action of hepatic duct and gallbladder bile of the cat, goat, rat, monkey, ox, pig, sheep and man.
The progress of sterilization of naturally infected gallbladder biles of rabbits, monkeys and man in vitro.
Are the antiseptic properties due to bacteriolytic antibodies?
Can agglutinins be demonstrated in bile?
Quantitative tests to determine the influence of reaction on the antiseptic effect of hepatic duct and gallbladder bile.
The rate of growth and the subsequent disinfection in rabbit bile.
The germicidal properties of hepatic duct bile in experimental acidosis and alkalosis.
Progress of sterilization in bile derived from hypercholesterolized rabbits.
Dilution and the addition of serum to hepatic duct bile.
The rate of growth and the subsequent disinfection in guinea-pig bile.
The rate of growth and the subsequent disinfection in dog bile.
The rate of growth and the selective antiseptic action in fresh and old cystic bile of the ox.
The growth of B. typhosus and Vibrio cholerae in human cystic bile at varying H-ion concentrations.

The problem of the typhoid carrier state, particularly the condition in which the prolonged sojourn of B. typhosus occurs in the gallbladder and the biliary passages, cannot be solved conclusively without giving due consideration to the biliary secretions. Some of the factors which are obviously responsible for the prompt localization of the typhoid bacillus, at least in the gallbladder of certain laboratory animals, have been discussed and illustrated in the preceding papers of this series.

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With the exception of Nichols,[1] and quite recently Beckwith,[2] no investigator interested in the experimental aspect of the carrier state paid sufficient attention to the bile. Nichols claimed that the bile of rabbits and guinea-pigs is strongly alkaline and therefore antiseptic. This conception is rather surprising and contrary to the commonly expressed belief in the literature that bile is a selective stimulating substance favoring the persistence of the typhoid bacillus. However, certain references supply data which confirm the conclusions of Nichols. The following questions, therefore, suggested themselves for investigation: 1. Is the bile of animals and man antiseptic, inhibitive or stimulating? 2. If antiseptic, is the sterilizing effect of bile due to the content of antibodies or due to chemical substances, or both? A study of the phase mentioned under (1) discloses two outstanding facts: First, we are unable to test with the present methods at our disposal the properties of bile in vivo. Second, in vitro experiments with bile must be considered test-tube artefacts. Our experiments in general support the view that bile in vivo is a favorable medium for the typhoid-paratyphoid-group of bacteria.

TABLE 1

Bacteriolytic Power (?) of Human Biliary Fistula-Bile for Typhoid Bacilli

Case	Treatment	Dosage of Bacilli per C c of Bile	Progress of Sterilization Day					
			1	2	3	5	10	15
L. St. 1917	Daily sodium Bicarbonate Magnesium sulfate and Carlsbader salt	500,000	+	+	+	+	+	+
		50,000	+	+	+	+	+	+
		5,000	+	+	+	+	+	+
		1,000	+	+	+	+	+	+
		100	+	+	+	+	+	+
		50	+	+	+	+	+	+
		25	+	+	+	+	+	+
		10	+	+	+	+	+	+
		5	+	+	+	+	+	+

REVIEW OF THE LITERATURE

Since 1884 nearly 50 authors have tested bacteriologically the properties of gallbladder and common-duct fistula bile of a variety of animals and also of man. The reports can be analyzed only with difficulty, on account of the diversity of methods and bile specimens employed. Some investigators tested fresh bile, others used old heated and sterilized biles in undiluted form, and still others added bile or bile

[1] J. Exper. Med., 1916, 24, p. 497.

[2] Thesis, 1920.

salts in varying amounts to liquid or solid nutritive substratums. Failure to appreciate the various factors which may alter the same bile specimen in reaction or in chemical composition and lead to a change in its properties as a culture medium, has produced an array of contradictory statements. Quantitative experiments have also been considered in a few instances, and biles collected in different localities have been tested with a variety of pathogenic organisms. Conclusions drawn from observations on ox bile have frequently been applied to other animal biles without the least experimental investigation. Based on the available data, let us see how far we are able to answer the question: Is bile antiseptic or not? Here again we meet with difficulties, because four different properties have been ascribed to the bile in vitro, namely, it favors the growth of the common bacteria, except pneumococci and streptococci, or it is indifferent or it can act as an inhibitive or even as a germicidal fluid on the bacterial proliferation. Some experiments have been recorded also, in which the rate of the inhibitive action invariably has led to a complete sterilization of the bile sample. We have observed the four phases in one bile sample and intend to consider the mechanism which produces the various end-results more carefully in the course of the analysis of our data. The findings published deal with the gallbladder bile of oxen, dogs, pigs, man, guinea-pigs and rabbits. Some observations made on fistula bile of rabbits and man are also recorded.

Unquestionably the most detailed studies were made with ox bile. Babes,[3] Dünschman,[4] Fisher,[5] Lagane,[6] Neufeld,[7] Ottolenghi[8] found it to be a growth-favoring fluid for typhoid, coli and other intestinal organisms, even the cholera vibrio. Bernabei[9] (not Corrado), Braun,[10] Charrin and Rogers,[11] Flu,[12] Gley and Lambling,[13] Leubuscher,[14] Nichols,[1] Vetrano,[15] Violle[16] report ox bile to be

[3] Berl. klin. Wchnschr., 1899, 36, p. 361.

[4] Ann. de l'Inst. Pasteur, 1909, 23, p. 29.

[5] Thesis, Bonn, 1894.

[6] Compt. rend. Soc. de biol., 1912, 73, p. 242.

[7] Neufeld, O., and Handel, O.: Arb. a. d. k. Gsndhtsamte, 1908, 28, p. 572.

[8] Centralbl. f. Bakteriol., O. I, 1911, 58, p. 120.

[9] Ibid., 1892, 12, p. 696; Atti dell Accad. Med. de Roma, 1890-91.

[10] Arch. d. Sc. biol., St. Petersburg, 1901, 8, p. 158.

[11] Compt. rend. Soc. de Biol., 1886, 3, p. 425.

[12] Geneesk. Tijdschr. v. Nederl. Indie, 1918, 58, p. 67.

[13] Rev. Biologique du Nord de la France, 1888, 1, p. 28.

[14] Ztschr. f. klin. Med., 1890, 17, p. 472.

[15] Centrlhl. f. Bakteriol. I, O., 1909, 52, p. 275.

[16] Ann. de l'Inst. Pasteur, 1912, 26, p. 381.

indifferent. Copeman and Winston,[17] Ehret and Stolz,[18] Ecker,[19] Fornet[20] and Jordan,[21] Obst,[22] Pies,[23] Tonney, Caldwell and Griffen[24] proved, however, conclusively, that this secretion is a poor culture medium, and when carefully tested exhibits regularly at least transitory inhibitive properties. Dog bile was found to be indifferent or slightly stimulating by Létienne[25] and Miyake,[26] while Ehret and Stolz[18] and Toida[27] noticed inhibition of bacterial growth. According to Leubuscher,[14] B. typhosus and B. coli grew well in pig's gallbladder bile.

Fresh human bladder bile can according to Babes,[8] Fränkel and Krause,[28] Fütterer,[29] Hirokowa,[30] Kramer,[31] Leubuscher,[14] Mieczowski,[32] Nichols,[1] Pies.[23] Toida[27] and others act as a good culture medium. Occasionally samples are found which inhibit strongly the representative organism of the colon-typhoid group (Pies,[23] Hirokowa,[30] Toida,[27] Copeman and Winston,[17] Ehret and Stolz[18] and Twort and Pastia.[33]

Guinea-pig's bile added to gelatine was found to be inhibitive by Copeman and Winston. Fresh alkaline fistulous bile of these animals is, according to Nichols,[1] strongly antiseptic. Vallardi and Bezzola[34] state that in guinea-pigs the resistance of the intestines to B. typhosus is not due to the pancreatic or biliary secretion.

Lange and Roos,[35] Rolly and Liebermeister,[36] Talma[37] and Nichols[38] reported the gallbladder bile of some rabbits as slightly or strongly inhibitive for B. typhosus, B. coli, etc. Vieillard-Baron,[39] Vincent[39] and Violle[16] found the antiseptic properties to be feeble or absent. Violle (p. 398), expresses himself on this subject as follows: "Il en est de même avec la bile de lapin ensemencée avec les bacilles typhiques, coli et le vibrion cholerique. Bien mieux, ce sera un milieu de predilection pour ces elements bacteriens, qui s'y developperont avec une rapidité beaucoup plus grande que dans toute autre substance nutritive." Nichols[1] tested the bactericidal properties of hepatic duct bile and found it strongly antiseptic for B. typhosus, B. paratyphosus A, B. coli and B. dysenteriae. The antiseptic action is, in his opinion, largely due to its alkalinity, which also explains his observation that Vibrio cholerae, an alkalophilic micro-organism, grew well in fresh rabbit bile. Aside from the carefully studied selective action of bile and bile salts on pneumococci, numerous reports indicate that one and the same sample may be antiseptic for one group and indifferent to another. Equally important and subject to further investigation is the rather superficially studied time rate of the inhibitive action or the velocity of the sterilizing process in various biles. Ehret and Stolz,[18] Fornet,[20] Pies,[23] and Ecker[19] supplied in this connection interesting data, which indicate that a small number of organisms is inhibited to a relatively greater degree than the large inoculum which is ordinarily chosen by the laboratory worker.

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[17] J. Physiol., 1889, 10, p. 213.

[18] Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 350; 7, p. 372.

[19] J. Infect. Dis., 1918, 22, p. 95.

[20] Arch. f. Hyg., 1907, 60, p. 134.

[21] J. Infect. Dis., 1913, 12, p. 326.

[22] J. Bacteriol., 1916, 1, p. 73.

[23] Arch. f. Hyg., 1907, 62, p. 107.

[24] J. Infect. Dis., 1916, 18, p. 239.

[25] Arch. de méd. expér. et d'anat. path., 1891, 1, p. 761.

[26] Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 479.

[27] Arch. f. klin. Chir., 1913-14, 103, p. 407.

[28] Ztschr. f. Hyg. u. Infectionskrank., 1889, 32, p. 97.

[29] München. med. Wchnschr., 1888, 35, p. 315.

[30] Centralbl. f. Bakteriol., O. I, 1909-10, 53, p. 12.

[31] J. Exper. Med., 1907, 9, p. 319.

[32] Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1900, 6, p. 307.

[33] Compt. rend. Soc. de biol., 1911, 71, p. 13.

[34] Abstr. in Off. internat. d'Hyg. Publique, 1918, 10, p. 921.

[35] Arb. a. d. Gsndhtsamte, 1915, 50, p. 57.

[36] Deutsch. Arch. f. klin. Med., 1905, 83, p. 413.

[37] Ztschr. f. klin. Med., 1901, 42, p. 354.

[38] Jour. Am. Med. Assn., 1917, 68, p. 958.

[39] Vieillard-Barron: Thesis No. 1038, Lyon, 1895. Ann. de l'Inst. Pasteur, 1908, 22, p. 341; Compt. rend. Soc. biol., 1916, 79, p. 580. Vincent, M. H., and Marbé: Compt. rend. Soc. biol., 1917, 80, p. 587. Vincent, M. H., and Fauré-Fremiet, E.: Compt. rend. Soc. biol., 1917, 80, p. 589. Vincent, Marbé and Muratel: Compt. rend. Soc. biol., 1917, 80, p. 675.

From this brief analysis it can be concluded that the results obtained by various workers with bile from the same type of animal and with the same bacterium are more or less at variance, but the majority of writers obtained results which indicate that bile in vitro may be more or less inhibitive, even slightly germicidal. Only Talma,[37] Violle[16] and Hailer and Ungermann[40] conducted in vivo experiments on the bactericidal properties of rabbit bile by the direct inoculation of B. typhosus into the gallbladder content of these animals. For example, Hailer and Ungermann noted regularly a development of the introduced bacilli in the gallbladder bile, irrespective of the number chosen, and 93% of their animals were found infected up to the 31st day after the inoculation. Less uniform were the results obtained by Talma,[37] who also injected typhoid and colon bacilli into the gallbladder of rabbits. The following points in his conclusions are of some interest: Rabbit bile contains a substance which inhibits most of the typhoid and colon bacilli The bactericidal property is probably the result of chemical action and the antiseptic action of rabbit bile varies in individual animals and at different time periods. If we accept, therefore, the conclusions, which are well supported by the recent careful studies of Jordan[41] and Ecker,[19] that bile is inhibitive even germicidal to a certain degree, we immediately have to answer the question: Is this antiseptic effect the result of (1) antibodies, or (2) chemical substances?

The old Italian and French medical literature deals extensively with the antitoxic properties and the virulence enhancing or depressing effect of the bile in general. The reader will find a complete review of this phase of the problem in a recent summary of Posselt.[42] On the other hand, definite statements with regard to the presence of specific antibodies in the bile are few and contradictory. Vincent[39] and his associates report on the demonstration of complement-fixing antibodies in a small percentage of rabbits immunized against B. typhosus, but no statement is made that the bactericidal properties of normal bile must be ascribed to such antibodies.

Most of the investigators conclude that the bacteriostatic and germicidal properties are the result of a chemical or of a physical action. In this connection we have to distinguish between an immediate action of the inhibitive substances on the proliferation of the inoculated bacteria and an indirect effect on the fermentative function of the micro-organisms (Roger[43]). The chemical properties and constituents of the bile, which are supposed to exert an inhibitive action on microbes are the reaction, the bile acids and their salts, the biliary lecithins and cholesterol, the biliary mucus, the lack of nutritive material, the surface tension and the photodynamic action of bilirubin.

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[40] Arb. a. d. k. Gsndhsamte, 1914, 47, p. 450.

[41] J. Infect. Dis., 1913, 12, p. 326.

[42] Ergebn. d. allg. Pathologie, 1915, 17, p. 783.

[43] Presse méd., 1913, 19, p. 137; Arch. de med. exper. et d'anat. path., 1913, 25, p. 430

In 1886 Charrin and Rogers[11] and in 1917 Nichols[1] suspected the reaction of the bile of oxen, rabbits and guinea-pigs to be largely responsible for their germicidal properties on typhoid, dysentery and paratyphoid bacilli. Their findings are in part supported by the observations of Sellards[44] made on pneumococci. The influence of dehydrated ox bile or a mixture of purified bile salts on bacteria has been studied with variant results by a number of workers. Sodium taurocholate (1-10% solutions) was found by Dünschman,[4] Mieczkowski,[32] Mosse[45] and Nicolle and Adil Bey[46] to be either stimulating or indifferent when added to solid or liquid culture mediums, while Bufalini,[47] Bunge,[48] Charrin and Rogers,[11] Lindberger,[49] Limbourg,[50] Leubuscher,[14] Löhlein,[51] Mac Conkey,[52] Maly and Emmerich[53] and Meyerstein,[54] report the free acids or their salts to be decidedly antiseptic. Sodium glycocholate was, in the experiments of Dünschman[4] and of Meyerstein[54] markedly inhibitive. Bassenge[55] considered the biliary lecithin to be a substance of considerable antiseptic power for B. typhosus. His observations that a 1% emulsion kills B. typhosus in from 30 to 60 minutes is not supported by the statements of Charrin and Rogers,[11] who found even a 10% solution to be innocuous. The opinion advanced by Daniel-Brunet and Rolland,[56] that 1% solution of cholesterol may check the proliferation of B. typhosus has recently been confirmed by Manfredi.[57] G. Mayer[58] found the biliary mucin to be inhibitive for diphtheria bacilli, but inactive for B. paratyphoid B, B. coli and B. typhosus. The lack of nutritive substances, particularly in fistular bile, perhaps the absence of vitamines, as suggested from the observations of Pies,[23] Meyerstein[54] and others, are factors which deserve a more thorough investigation in the future. Decidedly novel and of considerable importance are, in our opinion, the recent suggestions offered by Larson[59] and associates, who ascribed the lytic effect of bile on pneumococci to be the result of the well-known low surface tension of this body fluid. The cytolytic action of bilirubin was found to be slight by Charrin and Rogers,[11] while Sellards[44] considered the photodynamic properties of the biliary pigments to be quite marked. Under the conditions prevailing in our experiments the action of these substances was negligible.

It has not been our purpose to investigate all the questions suggested by the review of the literature, but rather to verify some of the published facts bearing on the carrier state and to determine by qualitative and quantitative tests on fresh and old bile samples of a large number of animals the rate of the germicidal action. Experiments have also been conducted to explain the factors responsible for the antiseptic effect, whether biologic or chemical.

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[44] Jour. Am. Med. Assn., 1918, 71, p. 1301.

[45] Ztschr. f. klin. Med., 1889, 36, p. 527; Ztschr. f. Hyg. u. Infektionskrankh., 1900, 34, p. 454.

[46] Ann. de l'Inst. Pasteur, 1907, 21, p. 20.

[47] Bufalini, G.: Boll. d. Soc. tra i cult d. sc. med. in Siena, 1884, 2, p. 142.

[48] Textbook of Physiol. and Path. Chem., 1902, p. 180.

[49] Upsala Läkaref. Förh., 1884, 19, p. 467.

[50] Ztschr. f. Physiol. Chem., 1889, 13, Nos. 1 and 2, p. 196.

[51] Klin. Monatsbl. f. Augenh., 1908, 46, p. 552.

[52] J. Hygiene, 1908, 8, p. 322.

[53] Sitzungsberichte d. K. Akademie d. Wissenschaften, 1883, 87, p.3.

[54] Centralbl. f. Bakteriol., O., I, 1907, 44, p. 434.

[55] Deutsch. med. Wchnschr., 1908, 34, p. 139.

[56] Compt. rend. Soc. de biol., 1911, 171, p. 298.

[57] Riforma med., 1917, 35, p. 849.

[58] Centralbl. f. Bakteriol., 1899, 25, p. 826.

[59] J. Infect. Dis., 1919, 25, p. 45.

METHOD OF STUDY

In the preceding paper of this series, we have described in detail the technic applied in keeping and collecting specimens of bile.

In the qualitative tests, immediately after titration, definite amounts of bile, usually 1 c c, were pipetted into cotton stoppered, serologic vials (7.5 x 2 cm.). For the quantitative tests triplicate sets of 5 c c of hepatic duct bile and 1 to 2 c c of cystic bile were aseptically distributed in pyrex tubes (100 x 12 mm.) with or without paraffin oil. One set of tubes was used for the determination of the changes in the H-ion concentration, a second for the same procedure after inoculation with bacteria and a third for plating.

When we began our tests a few years ago, we inoculated the bile samples with one loopful (2 mg. loop, 22 gauge wire made on a No. 2 Czaplewski standard rod) of a 24-hour peptic digest broth culture averaging 800,000 organisms for B. typhosus. In later experiments 1 loopful of a 1:1,000 or a 1:10,000 dilution of the same broth culture, approximating from 1,000 to 100 organisms, was used. The seeded tubes were always incubated at 37 C. The progress of the growth or the degree of the sterilization was followed by transferring at regular intervals of 2 hours 1 loopful of bile on to agar slants. It is self-explanatory that the proper inoculation of the bile samples was controlled by streaking an agar slant immediately on seeding of the tubes. Subcultures were made on peptic digest agar P_H^+ 7.4.

For the quantitative tests, 5 c c of bile were inoculated with 0.1 c c of a 24-hour broth culture diluted with distilled water, 1:10,000 or 1:100,000. These dilutions on the average approximated 300 to 500 organisms per 0.1 c c. At varying time intervals 0.1 c c amounts of seeded bile were plated either directly or in progressive saline dilutions. Invariably the determinations of the H-ion concentrations were made at the same time. In the quantitative tests the tubes were incubated in an open water bath or in a desiccator over $CaCl_2$ in an incubator.

A modification of the Buxton[60] method as applied to the determination of the bactericidal properties of serum was also used in some of our quantitative experiments. The procedure was as follows: A 24-hour agar slant culture was washed off with distilled water and standardized to 500,000,000 organisms per c c; 0.1 c c of the dilutions ranging from 1:10 to 1:1,000,000 were transferred into 1 c c lots of bile or serum. After an incubation of 5, 12, 24, etc., hours loop transfers were made on agar slants.

Suitable controls for each experiment, whether noted or not, were treated in exactly the same manner as the biles. The controls were both peptic-digest

[60] J. M. Res., 1905, 13, pp. 305 and 431.

broth P_H^+ 7.4, 0.01% and Witte's peptone solution P_H^+ 7.4, and according to the type of the experiment either distilled water or 0.85% saline solution.

In the quantative test, salt-free buffered broth, according to Dernby and Avery,[61] with hydrogen-ion concentration corresponding to the initial reaction of the biles, namely P_H^+ 7.6 and P_H^+ 8.6, was chosen. Some tubes of this medium were stratified with oil. We failed to note an inhibitive influence of this substance on the growth of the bacteria employed in our experiments.

Representative cultures of B. typhosus (K. and C.), B. paratyphosus A and B, B. dysenteriae, Staphylococcus and other cultures, were chosen which grew better in an alkaline than in an acid medium. In order to check the behavior of the bacteria in a general manner we selected the alkalophilic Vibrio cholerae.

An attempt was made to adapt the B. typhosus to an environment of bile by successive passage through ox bile, but this method was discontinued because no difference in adaptability could be noted. It is generally known that B. typhosus inoculated directly into the gallbladder grows luxuriantly. When such infected bile samples were used for the seeding of hepatic duct bile, it gave no evidence of having acquired increased resistance by its previous sojourn in the gallbladder.

In recording the results of growth on the slants the colonies were counted if they did not exceed 100, otherwise they were noted as positive. The plates prepared in the quantitative investigations were counted after 36 to 48 hours' incubation.

Our tests are divided into two groups, the qualitative and the quantitative. Having established the existence of an antiseptic action of certain bile specimens, we investigated the factors responsible for this property, dealing in the first place quantitatively with the question of bacteriolysins and immune bodies, and secondly, with the chemical aspect of the germicidal substances.

THE QUALITATIVE GERMICIDAL ACTION OF HEPATIC DUCT AND GALLBLADDER BILE OF RABBITS, GUINEA-PIGS DOGS, CATS, GOATS, RATS, MONKEY, OX, PIG, SHEEP AND MAN

Irrespective of initial H-ion concentration of the hepatic duct bile specimen derived from 45 rabbits and 10 guinea-pigs, the various strains of B. typhosus were unable to remain viable for more than 96 hours, if 1 c c lots were used for the tests 5 to 8 hours after collection. On the other hand, gallbladder bile of the same animals that was seeded in the same manner constantly gave positive subcultures for more than 10 days. Hepatic duct bile of rabbits therefore differs fundamentally with regard to its germicidal properties from true gallbladder biles.

The diet apparently influences in a slight degree the sterilizing effect of hepatic duct bile. Base-forming diets, for example, hay, oats, and carrots or cabbage produce a bile which destroys the ordinary stock typhoid strains (K and C) in from 48 to 196 hours. Another strain, more readily adapted to environmental influences, remained viable for

[61] J. Exper. M., 1918, 28, p. 345.

periods exceeding 72 to 156 hours. Acid-forming diets, oats and bread, prolonged occasionally the viability for several days. Heating of the hepatic bile specimens for from one-half to one hour at 56 C. shortened the period of disinfection. Slight differences in the rate of action were apparent when 2 c c or 4 c c samples were seeded instead of 1 c c specimens. The rate of lethal action was extended for from 2 to 5 days. With the exception of strain K, a small number of typhoid bacilli (100-200 bacteria) was more rapidly destroyed than the usual number of several thousand. This observation is quite in accordance with the findings of Ehret and Stolz,[18] of Fornet [20] and of Pies.[23]

A selective action of the germicidal substances of rabbit hepatic duct bile on various bacteria was also noted. It is most unfortunate that technical difficulties make it impossible to collect a sufficient amount of pure cystic bile for similar extended tests, but it is evident from the few data collected that the selective action is probably entirely absent or at least not as striking as is the case for hepatic duct bile. Paratyphoid bacilli behave similarly to B. typhosus; B. coli is somewhat more resistant; while Vibrio cholerae and Staphylococcus aureus must be looked on as somewhat better adapted species for growth and resistance in fistula bile of rabbits kept on mixed or on acid-forming diets. It should, however, be emphasized that even these organisms cannot lead to an indefinite existence on this type of bile, because subcultures can rarely be found positive after 10 days of incubation. The composition of the bile as a result of diet influences the selective action of Staphylococcus and Vibrio cholerae to slightly more marked degree, and variant results are more frequently encountered than with B. typhosus. The dysentery bacilli are considerably less resistant to the germicidal action; they rarely survive, even if inoculated in large doses. The only exception was the observation made on a bile specimen collected from fasting animals in which the bacilli lived for a period of 36 hours.

Guinea-pig hepatic duct bile exhibits greater disinfecting properties against the same bacteria than rabbit duct bile. The time rate of lethal action is shortened to 36 and 48 hours and even for Vibrio cholerae it never exceeds 5 days. Staphylococcus occasionally remains viable, even when transferred to this bile in rather small numbers. Otherwise the action seems to follow the same course as in the case of the hepatic duct bile of rabbits. It is usually impossible to obtain from guinea-pigs operated on for a common duct fistula sufficient cystic bile for a bacteriologic study. We therefore tested a large series of gallbladder biles collected from various necropsy examinations on animals submitted to anaphylaxis tests or those which succumbed to suffocation in a shipment. Most of the samples stood at room temperature for 24 to 48 hours, while the set of specimens collected from suffocated animals was inoculated and tested immediately.

Gallbladder bile specimens which appeared physically normal destroyed typhoid bacilli in 24 to 28 hours, that is to say, in a somewhat shorter time interval than hepatic duct bile. Three specimens which appeared colorless and physically changed acted more slowly in from 48-96 hours. The addition of blood prolonged the time rate of lethal action for over 240 hours. Fresh bile of suffocated guinea-pigs was not bactericidal for B. typhosus during the observation period of 400 days, but destroyed Vibrio cholera in 216 and 312 days, respectively. Old gallbladder bile of guinea-pigs may be antiseptic for cholera vibrios in a somewhat shorter time interval than hepatic duct bile. A few biles inoculated simultaneously while pipetting the samples into test tubes, and incubated at once showed a viability for B. typhosus of from 5 to 6 days.

In conclusion, we can state that our studies with bile specimens of rabbits and guinea-pigs have demonstrated that hepatic duct bile collected from rabbits and guinea-pigs in open tubes is antiseptic for B. typhosus, B. paratyphosus A, B. dysenteriae, B. coli, Vibrio cholerae, and Staphylococcus aureus. Pure, fresh cystic bile of rabbits and of suffocated guinea-pigs either lacks this property entirely or shows it in a less marked degree. The action is selective and apparently influenced by the diet. But we desire to emphasize the fact that the time rate of lethal action has never been less than 48 hours for the B. typhosus in hepatic bile of rabbits or 36 hours for that of guinea-pigs. Our observations made on a large series of animals differ therefore materially from those reported by Nichols,[1] who claims that with the exception of Vibrio cholerae one loopful of B. typhosus, B. coli, etc., was regularly killed by duct bile in 24 hours. He failed to state in his first paper whether this germicidal action is only to be attributed to the common duct fistula bile or to the cystic bile or to both. After we had called his attention to our results, he corrected this impression by stating that "the effect (antiseptic) was first noted with bile from a common duct fistula. It is not so definite, especially in the case of the rabbit, with bile from the gall-bladder, which is less alkaline and contains more solids than bile directly from the liver."[38] In subsequent paragraphs we shall explain the factors responsible for the differences between Nichols' and our observations, and discuss in detail his conception that the reaction of the bile is responsible for the germicidal action.

Pure gallbladder bile of rabbits in vitro has no antiseptic properties. It may be inhibitive to a slight degree or a poorer medium than broth and may even destroy after prolonged incubation (9 to 20 days) at 37 C. a small number of typhoid bacilli, but it certainly exhibits a different action than does the hepatic duct bile. As the conclusions of Nichols are based on the observations with the latter, his arguments that the bile is an important factor in the development of typhoid carriers are materially deprived of support by our own data, a point which will be discussed from various other angles in the course of this study.

The same differences in the sterilizing properties of cystic and hepatic duct bile as discussed for the rabbit and guinea-pig are also present in the dog. Most of the bacteria remained viable in the gallbladder bile for periods extending over 10 days. On the other hand, 4 of the 7 dogs which had gallbladder biles acting indifferently on bacteria, produced antiseptic fistula biles. Typhoid bacilli inoculated in large numbers rarely survived an incubation period of 120 hours. The colon bacillus and staphylococcus resist an exposure for more than 240 days. Heating of the bile to 56 C. for 30 minutes has no influence on the antiseptic properties. Three other dogs furnished hepatic biles which were bactericidal for dysentery bacilli and in one instance also for a small number of typhoid bacilli. The dogs were all kept on a meat and bread diet and had received inoculations of sodium taurocholate for reasons already explained in the preceding paper of this series. We are quite aware of the fact that the treatment with bile salts may have produced an abnormal hepatic bile, and that we were actually dealing in these experiments with test tube artefacts. This is probably true to a certain extent, but on further analysis these observations offer some explanation for the antiseptic properties of bile in general, namely, sodium taurocholate in an alkaline solution is destructive for B. typhosus and other organisms. Proper attention must be given to these dog experiments in interpreting the factors responsible for the bacteriostatic and antiseptic properties of bile. Natural gallbladder bile of dogs has bacteriostatic properties and may be considered a fair medium for the development of B. typhosus, B. coli, etc. According to Toida[27] they may remain viable for from 40 to 70 days.

The differences noted in the germicidal behavior of hepatic duct and cystic bile of rabbits, guinea-pigs and dogs suggested some experiments on other animals. With the same technic we failed to detect in cats, goats, rats and monkeys, any bacteriostatic or antiseptic action of hepatic duct bile. At least qualitatively, the liver bile acts similarly to the gallbladder bile. Fresh unheated gallbladder bile of oxen, pigs and sheep collected in December, retained large numbers of viable typhoid bacilli for more than 10 days. This crude method of testing the properties of a bile sample gave the impression that the bile of these animals in comparison with the hepatic duct bile of rabbits, guinea-pigs and dogs is an indifferent or even a good medium for the growth of B. typhosus. Quantitative tests to be described later gave us a better insight into the bacteriologic properties of the bile of these animals and contributed also to the understanding of the factors and the mechanism of the striking lethal action of certain hepatic bile samples.

The few tests conducted with human bile specimens deserve brief consideration. It was not our intention to verify the carefully conducted experiments of Fränkel and Krause,[28] Hirokawa,[30] Toida [27] and others, but we were fortunate in having access to biliary secretions which were collected intra vitam, and to a case of biliary fistula, on which we were enabled to study the influence of an alkaline therapy on the germicidal action of bile.

The gallbladder specimens were obtained either from cholecystectomized bladders or by aspiration at laparotomy. The samples were sterile and were primarily used for the determination of the H-ion concentration. Typhoid bacilli grew well in these secretions and remained viable for more than 10 days; in the only sample tested with the Vibrio cholerae complete sterilization took place on the eighth day. This observation, which confirms that of Toida and which may have some bearing on the cholera-carrier problem, deserves further and more extended investigation.

The hepatic duct bile for our alkaline therapy experiment was collected from a man, 55 years of age, who developed subsequent to cholecystectomy and kinking of the cystic duct, a postoperative fistula. The bile specimens were on several occasions infected with B. coli aerogenes. The patient felt completely comfortable only when taking daily Carlsbader salt; the secretion from the fistula was considerably more mucoid and the jaundice more marked during the few days we excluded this medicament. For a period of several weeks daily doses of 6 gm. of sodium bicarbonate were administered; to this was added magnesium sulfate or Carlsbader salt. We have already discussed in the preceding paper the fact that only slight changes in the reaction were noticeable and that the bile was nearly always neutral to phenolphthalein or 2.2 alkaline to lacmoid with a P_H^+ of 8.0. This bile never exhibited, when repeatedly tested, any germicidal properties, as is shown in table 1. Even such small numbers as 5 to 10 typhoid bacilli grew freely in this medium and remained viable in large numbers for over 15 days. Human fistula bile collected from an intensively alkalinized patient is not antiseptic in test-tube experiments, and it appears to us most unlikely that this property is present in the body. Our observations on rabbits, to be detailed, negative such an assumption.

Qualitative tests with hepatic duct bile collected in test tubes exposed to air from rabbits, guinea-pigs, dogs, cats, monkeys, goats and man, as well as gallbladder biles of the same animals in addition to that of oxen, sheep and pigs, have demonstrated that with the exception of guinea-pig bile, no true germicidal effect was demonstrable in the first 24 hours after the inoculation. The time rate of lethal action varied between 36 and 240 hours for fistula bile of rabbits and dogs, and for gallbladder biles exceeded many times the longest period noted for the hepatic duct biles. There is a significant selective action on B. dysenteriae in comparison to Vibrio cholerae, but one is unable to state whether this is similar to the one commonly observed with chemical or biologic bactericides, or whether it is due to an entirely new property. It was noted that in hepatic duct bile of rabbits an appreciable increase of organisms occurred between the 5th and 12th hour after the inoculation of the specimen with a few bacteria, and yet transplants from such tubes at the 24th and 48th hour gave complete sterility. Quantitative tests alone can explain these differences and possibly assist in the understanding of the cycle of events which take place in the bile removed from the body and placed in an open test tube.

THE PROGRESS OF STERILIZATION OF NATURALLY INFECTED GALLBLADDER BILE OF RABBITS, GUINEA-PIGS, MONKEYS AND MAN IN VITRO

In the preceding paper we have called attention to the low H-ion concentration of gallbladder carrier bile of rabbits, and we explained this observation by the fact that the gallbladder wall is seriously damaged and that the infected secretion is mainly hepatic duct bile. A study of the noninfected hepatic duct bile was therefore essential to a clear understanding of the relationship of bile to the carrier state, a phase of the problem which had been qualitatively dealt with in the preceding paragraphs. For this purpose a large series of gallbladder biles collected from rabbits in various stages of infection was tested qualitatively over periods extending to two months. A few unselected observations are shown in table 2.

Nearly one-half of the rabbit gallbladder biles infected for varying time intervals became sterile in from 4 to 10 days. According to the available notes, these biles were limpid, light green and contained macroscopically no pus or carbonate débris. The number of viable typhoid bacilli determined by plating frequently exceeded one million per c c. The other half of the carrier biles were all more or less purulent or distinctly viscid, some forming, on standing, a heavy sediment of greenish, sand-like carbonate débris. In these samples B. typhosus or B. paratyphosus remained viable for more than 30 days, irrespective of the agglutinin content. The physical consistency of the bile, and not the duration of the carrier state, governed the persistence of viable organisms. In view of these results it is evident that removal of the bile from the gallbladder and its exposure to air may have produced antiseptic substances. In all probability these did not, and would not, have developed in the same time interval in the gallbladder in vivo. Progressive decrease in the H-ion concentration in pus-free bile specimens is probably one of the factors responsible for this action. In purulent carrier biles in vitro the greater viability of B. typhosus is probably explained by an absence of a decrease in the H-ion concentration as a result of constant CO_2 formation by the pus cells in the sediment, or later the formation of lactic acid as a result of a destruction of cells on standing. It is of interest in this connection to recall that in the course of these tests we became convinced that removal from the animal body is conducive to alteration in the physical and chemical composition of the bile, and that exposure to air produces an unsuitable medium for B. typhosus. It also became apparent that it is obviously wrong to draw conclusions relative to the action of rabbit bile on B. typhosus when the secretion is exposed to air. The time rate of lethal action was probably a variant factor on account of the different composition and content of bile salts.

The observations made on rabbit bile were confirmed by similar tests with guinea-pig and monkey bile specimens. Dog biles were

TABLE 2

PROGRESS OF STERILIZATION OF NATURALLY INFECTED GALLBLADDER BILES OF RABBITS, GUINEA-PIGS, MONKEYS AND MAN

Rabbit	Method of Infection and When Killed	Agglutination Reactions		Number of B. Typhosus (Polyvalent) per C c of Bile	Incubated at 37 C. Found Sterile on
		Serum	Bile		
1593	Intravenous, killed 120 hours after injection	40,000	100	50,000 per 8 c c no pus	19th day
1595	Intravenous, killed 10 minutes after injection	1:10++	1:10++	1334 per 0.5 c c no pus	10th day
1609	Carrier, 48 days' duration	1:10,000	<1:10	>5,000,000 per c c pus	21st day
1630	Carrier, 117 days' duration	1:8000	1:10++	>5,000,000 per c c pus	>20th day
1591	Intravenous, 96 hours after injection, killed	1:60,000	1:20+++	1,420,000 per c c no pus	21st day
1715	Intravenous, 48 hours after injection, killed	1:10+++	<1:10	140,000,000 per c c	>20 days
1720	Intravenous, 10 minutes after injection, killed	<1:10	<1:10	30 per c c	>20 days
800	Carrier for 84 days, chloroformed	1:1000	....	About 50,000 per c c	4 days
805	Carrier for 84 days, chloroformed	1:4000	....	>1,000,000 per c c	4 days
806	Carrier for 83 days, chloroformed	1:6000	....	>1,000,000 per c c	4 days
912	Carrier for 64 days, died	1:1000	....	>1,000,000 per c c	>60 days
926	Paratyphoid B carrier 120 days, died	1:4000	....	>1,000,000 per c c	>20 days
954	Typhoid carrier for 17 days, died	1:80,000	....	>1,000,000 per c c	>20 days
972	Typhoid carrier for 10 days, chloroformed	1:400	....	>1,000,000 per c c	5th day
963	Typhoid carrier for 272 days	1:200	1:10++	12,000,000 per c c	9th day
1028	Typhoid carrier for 29 days	....	>1:20	27,000 per c c	>30 days
1049	Typhoid infection 19 days; purulent, blood, coagula, etc.	>1:20,000	1:600	14,000,000 per c c	>60 days
1083	Typhoid infection, 7 days; died	1:10,000	1:100	63,000,000 per c c	12th day
1160	Typhoid carrier, 66 days; purulent	1:2000	1:10	11,800,000 per c c	>44 days
1164	Typhoid carrier, 65 days	1:2000	....	317,000,000 per c c	5th day
1491	Typhoid infection 1 day	1:20,000	....	5,000,000 per c c	5th day
			Guinea-Pigs		
322	Typhoid infection 7 days	1:100	....	>1000 per c c	2d day
326	Typhoid infection 21 days; died	....	....	∞ B. typhosus	2d day
			Monkey		
S.I. Macacus rhesus	Carrier for 14 days; light green, thin bile	1:8000+++	1:10	Innumerable	5th day
			Human		
Mrs. Bl.	Cholecystectomy following typhoid fever (2/26/18)	....	....	30,000,000 per c c	20th day incubator dried out; room temp. >79 days; in ice chest >79 days
			Dogs		
3	Intravenous, 24 hours after infection, killed	<1:10	<1:10	30 per c c	Exposed to air 21 days; paraffin oil >30 days
6	Intravenous, 4 hours after infection, killed	<1:10	<1:10	2 per c c	Exposed to air >30 days; paraffin oil >30 days

always viscid and behaved like true gallbladder biles. The only human bile examined was purulent, and registered, after exposure to air, only minimum changes in the reaction, which naturally favored the viability of B. typhosus therein. The draining gallbladder of the patient was found free of typhoid bacilli 28, 36, and 50 days after the specimen for the test-tube experiments was collected, and yet in vitro the organism continued to flourish. We mention this fact merely as a paradoxical curiosity which again illustrates our belief that test-tube experiments are frequently misleading artefacts.

IS THE ANTISEPTIC EFFECT THE RESULT OF BACTERIOLYTIC ANTIBODIES?

The observations of Buxton,[60] Bull,[62] and others recently confirmed by Teague and McWilliams [63] have shown that normal rabbit serum is capable of killing large numbers of typhoid and paratyphoid A bacilli. The close relationship between the blood and the secreted bile in the liver shown by the experiments dealing with the passage of microorganisms from the interlobular veins to the bile capillaries suggested carrying out some tests to determine the presence of bacteriolysins. It was also necessary to settle more satisfactorily the questions dealing with the elimination of agglutinins and antibodies in the bile.

According to Vincent [16] and his pupils, who diligently investigated the presence of complement-fixing antibodies and the so-called "substance anti-sensibilisatrice" in the bile of typhoid immune rabbits, bacteria thrive in the bile of such animals on account of the failure of antibodies to reach the gallbladder or because the antibodies are not persistent on account of the continuous flow and renewal of the secretion. The latter supposition is supported by the observation that the fixing bodies are either absent (in 2 only out of 9 biles examined) or disappear rapidly from the viscus. Forster and Kayser state that bile of immunized rabbits occasionally exhibited bactericidal properties in the peritoneal cavity of guinea-pigs when used in a Pfeiffer bacteriolysin test.

The results of our tests with fresh hepatic duct bile of normal rabbits kept on different diets are given in table 3. We also tested the bile of some immune rabbits and guinea-pigs. With the exception of the latter, which apparently destroyed uniformly 1,000,000 organisms in from 24 to 36 hours, the results were practically identical with those illustrated in the table and are therefore not recorded in detail.

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[62] J. Exper. M., 1915, 22, p. 475.

[63] J. Immunol., 1917, 2, p. 93.

Rabbits' hepatic duct bile is capable of destroying any number of living typhoid bacilli in from 5 to 7 days when kept in test tubes exposed to air. Irrespective of the bacteria inoculated, growth was always present with as small a number as 5 organisms, although it rarely extended over the 48th hour.

There is an absence of a graded diminution in the number of viable organisms as ordinarily seen in quantitative biologic tests for serum bacteriolysis; on the contrary, a sudden destruction of a large number of living typhoid bacilli takes place on the 5th day. The serum of the same rabbit is capable of destroying 1,000,000 bacteria in 1 c c of serum in 24 hours. Bacteriolytic antibodies in an immunologic sense are not active in rabbits' hepatic bile, and other factors probably explain the interesting bactericidal properties of this secretion.

The same conclusion can be applied to the bile of other animals, namely, the antiseptic property is not due to bacteriolysins or "a substance sensibilitrice."

CAN AGGLUTININS BE DEMONSTRATED IN BILE?

In this connection we investigated the readily demonstrable immune bodies in the hepatic duct and cystic bile of normal, immune and infected rabbits and guinea-pigs. The findings of agglutinins in the gallbladder biles of immunized rabbits (also guinea-pigs?) reported by Cantani [11] are contradicted by the observations of Forster and Kayser,[64] Venema [65] and Schöbl on rabbits and by Stäubli,[66] who studied the subject in guinea-pigs. Stäubli pointed out that bile from cadavers collected many hours after death may contain agglutinins and that the collection of bile from highly immunized animals (guinea-pigs) must be conducted with great care in order to avoid the admixture of traces of blood, which may readily produce positive agglutination reactions in the otherwise agglutinin-free bile. The presence of clumped bacteria in the gallbladder content is, however, commented upon by Cushing,[67] Richardson,[68] Doerr [69] and others.

In our tests we followed the customary technic, using formalinized standardized suspensions both for the bile and serum samples.

[64] Munchen. med. Wchnschr., 1905, 31, p. 1473; 52, p. 1476.

[65] Berl. klin. Wchnschr., 1906, 43, p. 99.

[66] Centralbl. f. Bakteriol., I, O., 1903, 33, p. 375.

[67] Bull. Johns Hopkins Hosp., 1898, 9, p. 91.

[68] J. Boston Soc. of Med. Sc., 1898-99, 3, p. 29.

[69] Centralbl. f. Bakteriol., I, O., 1905, 39, p. 624.

Seven normal rabbits had neither in the hepatic duct nor in the gallbladder bile agglutinins above the dilution of 1:10. The cystic bile obtained by laparotomy from 3 immunized rabbits in a series of 13 tested, agglutinated formalinized typhoid bacilli in a dilution of 1:60 or even 1:100. The remaining 10 agglutinated in a dilution of 1:10 or not at all. Bile derived from 10 rabbits which had been chloroformed in various stages of immunization or recovery from infection in 2 animals gave a reaction as high as 1:200 and 1:1000, respectively. Three recovered carriers with marked thickening of the gallbladder wall agglutinated in a dilution of 1:400-600. One immunized animal, which died from an intercurrent infection, was necropsied 8 to 10 hours after death and furnished a cystic bile which clumped typhoid bacilli in a dilution as high as 1:1000. Six normal and 8 immunized guinea-pigs gave hepatic and cystic duct biles which were free from agglutinins irrespective of the serum agglutination titer that they possessed. Our observations confirm those of Foster and Kayser[64] (p. 1476) and indicate that 25% of the immunized rabbits or recovered carriers may possess agglutinins in the bile. The source of these immune substances cannot be determined with certainty. Some of the rabbits with positive bile specimens had liver injuries and showed the lesions of a chronic cholecystitis or had indications of ascites on account of renal injury caused by a preceding typhoid infection. The degree of the agglutination was entirely independent of the serum agglutinins. The absence of such antibodies in the hepatic duct bile is ample proof, in our opinion, that a true secretion of agglutinins in the same sense as we accept it to be the case for the milk does not take place for the bile, at least in rabbits and guinea-pigs. This fact supports our contention that the antiseptic effect of bile cannot be the result of true, biologically demonstrable antibodies.

A few words on the agglutinins of carrier biles should, however, be added in this connection. An extensive series of gallbladder bile derived from infected animals in various stages of the infection were at our disposal. A total of 31 rabbits was examined; 25 animals were chloroformed, 2 laparotomized and 5 specimens were collected after death. Twenty-five, or 78% of the samples, gave distinct agglutination reactions in dilutions above 1:10, nearly 56% of these biles reacted even in a dilution of 1:100 and above. The strongly positive biles were derived from rabbits, which showed gallbladders in a state of stasis, enormously distended and filled with a pigmentless, slightly purulent bile. The gallbladder wall was often diphtheritic or in old carriers thickened by the inflammatory process. In others the agglutinin content was due to a slight contamination with blood, and in still others the collection many hours after death must be considered responsible for the presence of agglutinins. The number of viable B. typhosus present, not the duration of the carrier stage, apparently influenced the antibody content. The correlation of the findings suggests, that prolonged biliary stasis in the diseased gallbladder due to obstruction of the cystic duct by sandlike carbonate débris or a severe necrotizing, inflammatory process in this viscus are conducive to the presence of agglutinins, and explains the regular finding of clusters of typhoid bacilli in the sediment derived from such biles. How far these antibodies influence the course and duration of the carrier state is difficult to say. As a rule, progressive repair of the inflammatory cholecystitis and hepatitis is shown by a return of the normal color of the bile and is usually accompanied by a marked diminution or even complete disappearance of the agglutinins irrespective of the presence of B. typhosus. Gallbladders with impaired function, irrespective of their agglutinin containing bile, more often remain infected for longer periods than the unobstructed ones.

QUANTITATIVE TESTS TO DETERMINE THE FACTORS RESPONSIBLE FOR THE ANTISEPTIC EFFECT OF HEPATIC DUCT AND GALLBLADDER BILE OF RABBITS, GUINEA-PIGS, DOGS, AND OXEN

From the review of the literature we gained the impression that the reaction, the bile salts, the biliary lecithin and cholesterin play either alone or in combination the rôle of disinfecting substances in bile specimens tested in vitro. The selective action and the variant behavior of the biles of the different animals may in part be the result of the composition of the secretion. This is particularly true with regard to the bile salts. According to Hammarsten [70] human, rabbit, monkey and ox bile contain mostly, and pig bile entirely, the sodium salt of glycocholic acid. On the other hand, in dog, sheep and goat, bile taurocholic compounds either predominate or are found exclusively in many specimens. The antiseptic properties of the various bile salts in different concentrations (a test-tube experiment) will be treated in a separate paper.

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[70] Lehrbuch der physiologischen Chemie., 1914, p. 390. Ergebn. d. Physiologie., 1905, 4, p. 1.

In this connection attention is called to the recent observation of Davis and Hain [71] and to our own, that rabbits' urine possesses a germicidal action, particularly against the organisms of the colon-typhoid group. The antiseptic properties are inconstant in that all urines do not possess equal action nor are all specimens of urine from the same rabbit of like potency. The action is influenced by diet, but is independent of the H-ion concentration of the urine. The nature of the substances responsible for these germicidal properties is as yet unknown. At the beginning of our studies on the antiseptic action of bile we were favorably inclined to suspect an analogy between biliary and urinary antisepsis, but subsequent tests did not support this view. In most of the experiments the urine specimen of rabbits or guinea-pigs, which gave a strongly antiseptic bile, permitted a free development of B. typhosus and allied organisms.

This and other observations stigmatized the reaction as the most important factor in the germicidal action of hepatic duct bile. As early as 1886 Charrin and Rogers [11] came to a similar conclusion and expressed their view in the following sentence (p. 426): "Est-ce à sa reaction que la bile en nature doit ce pouvoir antiseptique moindre que celui de quelques unes de ses élements." Nichols [1] was even more convinced that the alkalinity of the hepatic duct bile of rabbits and guinea-pigs was responsible for its germicidal action because the latter disappeared on neutralization. The importance of the reaction was also recognized by Meyerstein [54] in his tests with various mixtures of bile salts; a neutral or slightly acid reaction favored the growth of B. typhosus, while an alkaline reaction was decidedly inhibitive.

In the early period of the research on immunity, von Fodor,[72] Emmerich [73] and others advanced the theory, based on observations made on rabbits infected with anthrax, that the bactericidal properties of the serum are closely connected with the alkalinity of the blood. The influence of the reaction is undoubtedly only secondary and plays only a subordinate rôle. This theory has therefore been entirely disregarded. The preliminary bile studies, which have given the desired information to conduct the quantitative tests reported below, are presented in the preceding papers. Having established the optimum H-ion concentration for the growth of B. typhosus and also its generation time, we studied

[71] J. Urolog., 1918, 2, p. 309.

[72] Arch. Hyg., 1886, 4, p. 129.

[73] Emmerich, E., and Wagner, G.: Centralbl. f. allg. u. path. Anat., 1916, 27, p. 433. Ztschr. f. Immunitätsforsch. u. exper. Therap., 1916, 24, p. 557.

in detail the changes which take place in the hepatic duct bile of rabbits and guinea-pigs on standing exposed to air or alkali. It was found that on account of the escape of CO_2, and possibly the absorption of ammonia, the H-ion concentration decreased progressively in 24 hours to a P_{H^+} of from 8.6 to >9.0; in a similar manner the titrable alkalinity increased to a marked degree. On the other hand, it was demonstrated that these changes could be retarded by the stratification of the biliary effluent with paraffin oil during the act of collection and during the experiments. We have stated in this connection that the reaction of hepatic duct bile in vitro exposed to air for even a short time differs materially from that present in the animal body. Similar changes in the reaction to a less marked degree occurred in the cystic bile and a final P_{H^+} over 8.4 was occasionally recorded. It was also pointed out that the diet and the biliary stasis provoked by starvation affect the H-ion concentration of the gallbladder bile.

These principles were applied to the study of the antiseptic effect of hepatic duct bile of rabbits and later extended to that of guinea-pigs, oxen and man. Definite amounts (usually 5 c c, cystic bile 1-2 c c) of fresh bile collected under oil, together with broth controls of a definite P_{H^+}, were seeded with 300-500 typhoid or dysentery bacilli or Vibrio cholerae and kept in a water bath at 37 C. At varying time intervals 0.1 c c amounts were plated either directly or in progressive dilutions in peptic digest agar. After 24-36 hours' incubation at 37 C. the plates were counted. It is customary to use the logarithm of the number of viable organisms in recording results of experiments on the rate of growth of bacteria. In the accompanying charts the logarithms of the viable organisms per 0.1 c c are plotted as ordinates against time intervals as abscissae. Bile samples, which show by plating active, vigorous growth, are never turbid; they may when exposed to air change their color to an olive-brown, but when stratified with oil they always remained smaragadine green. It was also noted that certain types of bacteria reduced the biliary pigment more readily than others.

From an extensive series of identical tests we select a few illustrative examples.

THE RATE OF GROWTH AND THE SUBSEQUENT DISINFECTION OF RABBIT BILE

Chart 1 illustrates the rate of growth of B. typhosus in the hepatic bile of a rabbit kept on an oat diet, compared with the one in buffered salt free broth. Similar experiments conducted with hepatic duct bile of rabbits kept on a mixed and on a cabbage diet gave identical results. Hepatic bile of rabbits, when tested in vitro exposed to air and alkali favors in the first 24 hours the growth of B. typhosus; later it acquires germicidal properties, which lead, irrespective of the diet of animals, to complete disinfection of this body fluid in 96 hours. This definite cycle of events, so well illustrated graphically, can be prevented by stratification of the hepatic duct bile with oil. Cessation of growth occurs in the stratified bile at the same time as in the broth, and the disappearance of viable typhoid bacilli progresses in a similar manner. As the typhoid bacilli inoculated in the bile specimens were grown in a broth of a reaction of P_{H^+} 7.0, transplantations to mediums of more alkaline reactions (P_{H^+} 8.4-8.6) were expected to produce a decided "lag."

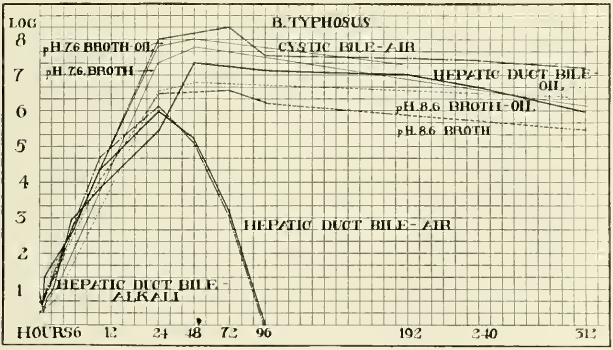

Chart 1.—Rate of growth of B. typhosus in the hepatic duct bile of a rabbit kept on an oat diet compared with growth of the bacillus in buffered salt-free broth.

If we assume for the present that the nutritive requirements are satisfactory for the growth of B. typhosus and recall that this organism has a distinct adaptability to changes in H-ion concentration; although it is more alkalo—than acidophilic, it appears not at all surprising that the initial growth in all the bile specimens is good. Once accustomed to the environment, the organisms continue to grow slightly or remain viable even when the reaction has undoubtedly reached an unfavorable point, above P_{H^+} 8.8. The acquired alkali tolerance of B. typhosus in milk illustrates this well. Test-tube experiments with a salt-free broth containing 1% of peptone with phosphate mixtures and sodium hydroxide have shown that the "alkali death point" for B. typhosus (K) in such a medium varies between P_H^+ 9.2 and 9.4. A substratum containing more peptones or colloids pushes the "death point" to P_H^+ 9.6 or even 9.8.

When bile is exposed to alkali, progressive decrease in the H-ion concentration following the escape of CO_2 may reach its lowest level in from 10 to 12 hours at 37 C.; yet the typhoid bacillus continues to grow only to be inhibited and destroyed 36 to 48 hours later. Other organisms may develop in the same environment for periods extending over 10 days. This phenomenon was called to our attention by the experiment illustrated in chart 2. The alkalophilic Vibrio cholerae grows favorably in rabbit bile covered by oil and is completely killed in the secretion exposed to air after an incubation time of 12 days. Cessation of growth takes place in all tubes inclusive of the broth control after 48 hours' incubation. The progressive disappearance of viable organisms proceeds slowly in the broth and bile tube stratified with oil, and more rapidly in the tubes exposed to air or alkali. The decrease in the tube kept in the desiccator is apparently interrupted after the 144th hour. In this experiment the tube remained unopened in the desiccator for 6 days and was found to contain a large number of viable vibrios on the 12th day of incubation. The reaction of this specimen

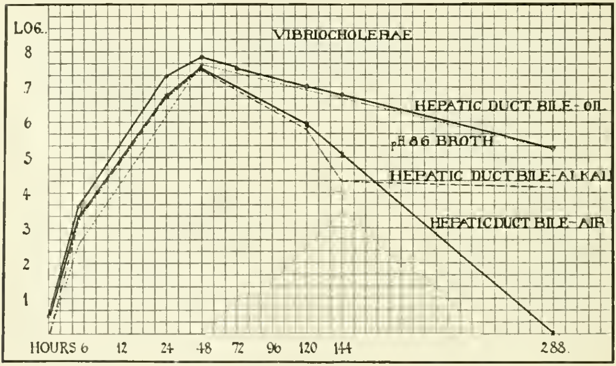

Chart 2.—Rate of growth of Vibrio cholerae in the hepatic duct of a rabbit.

differed only slightly from that exposed to air, and yet the spirillae remained viable. The absence of germicidal properties for Vibrio cholerae in bile kept in a sealed desiccator is rather difficult to explain. The nature of the factors operative on the bile in this environment, in contrast to that exposed to air, is not clearly understood. Three possibilities suggest themselves: (1) absorption of water and concentration of the bile (accurate quantitative tests were not made but casual observations failed to support this particular contention); (2) continuous absorption of the ammonia produced by the growth of the organisms; and (3) the prevention of ammonia absorption from the air.

Old bile exposed to the air at room temperature or in the ice-chest is antiseptic the first 24 hours for B. typhosus and B. dysenteriae Shiga. This observation explains the results of Nichols and our own in the qualitative series. Rabbit hepatic duct bile collected in open tubes and kept at room temperature even for 24 hours will exhibit strongly bactericidal properties; the inoculated bacteria are unable to grow and are progressively destroyed after 15 hours of incubation. Such biles can be partially rejuvenated by saturation with CO_2 and subsequent stratification with oil. Various bacteria are enabled to grow at least for 15 hours but subsequently germicidal substances make their appearance and sterilize the hepatic bile in 48 hours when exposed to air, or in from 192 to 216 hours when stratified with oil.

A similar cycle of events takes place in bile samples which are neutralized by the addition of HCl or lactic acid. These experiments were merely undertaken to verify the observations of Nichols.[1] It is quite obvious that the addition of strong acids, such as hydrochloric and sulphuric as recommended by this worker, may, aside from neutralizing the carbonates, materially change the chemical composition of the secretion and produce conditions which never occur in the animal body. Neutralization of fresh and old bile, which is germicidal in 24 to 96 hours, produces a hepatic duct bile suitable for the growth of a variety of bacteria. Invariably the antiseptic properties manifest themselves in the neutralized biles in from 1 to 8 days later than in the untreated ones. Bile specimens which are neutral show a decline in the H-ion concentration probably due to an escape of CO_2 or to an absorption of ammonia. The inoculated types of bacteria, however, grow well and remain viable for over 10 days. Such biles are antiseptic for Vibrio cholerae. The change in the H-ion concentration is therefore in all probability only one of the many factors which is altered under the influence of the neutralization process.

THE GERMICIDAL PROPERTIES OF HEPATIC DUCT BILE IN EXPERIMENTAL ALKALOSIS AND ACIDOSIS

In the preceding paper we discussed the experiments which were undertaken with the intention of determining the H-ion concentration of hepatic duct bile under the influence of an experimentally produced alkalosis or acidosis. It was stated that the reaction of this secretion is rarely changed on account of the feeding or the injection of $NaHCO_3$ or HCl. It was, however, shown that the reaction of the cystic bile of rabbits may be distinctly influenced by the same treatment with these chemicals or by fasting. In the discussion of the qualitative tests reported in the preceding paragraphs of this paper, it was shown that acid-forming diets may produce a hepatic duct bile which becomes only germicidal after 96 to 168 hours' standing at room temperature. These facts naturally suggest some experiments in order to prove or to refute the contention of Nichols that an alkaline therapy may be of value in preventing and curing typhoid gallbladder carriers. Animals kept on acid-forming or base-forming diet were in the course of the experiment treated with alkalies, and the hepatic duct bile collected before and after the administration of the chemicals was submitted to qualitative tests.

The intravenous injection of sodium bicarbonate produced in one rabbit a hepatic duct bile in which B. typhosus was killed. From another rabbit a similarly active bile specimen was collected after the administration of 2 gm. of $NaCO_3$ intravenously and of 5 gm. by stomach tube. An animal kept on a base-forming diet for 16 days and intensively alkalinized before the operation furnished a hepatic duct bile and a cystic bile which differed in no respect from those obtained from rabbits kept on a base-forming diet alone. These few experiments are suggestive, but further tests on a large series of animals are necessary before final conclusions can be reached. It is quite obvious from the observations already reported that we are dealing in those germicidal tests with test-tube experiments, and that we have no knowledge of the physiologic or pharmacologic action of alkalies on the rabbit organs and tissues or on the composition of the secreted bile. Observations to be reported in a subsequent paper indicate that alkalinization of typhoid infected rabbits may act indirectly in preventing the accompanying acidosis and not through their immediate action on the organisms themselves. It is in this sense that we feel the experiments of Nichols may finally be interpreted, when all the factors concerned have been fully analyzed.

TABLE 3

BACTERIOLYTIC POWER (?) OF HEPATIC DUCT BILE OF RABBIT FOR TYPHOID BACILLI

Rabbit	Diet	Dosage of Bacilli per C c of Bile	Progress of Sterilization Days							Remarks
			1	2	3	4	5	6	7	
1418 Serum agglut: 1:5 Bile agglut: <1:5	Mixed diet	710,000	+	+	+	+	0	..	..	Open in tube with cotton stopper
		300,000	+	+	+	+	0	..	..	One c c of serum destroyed 1,000,000 in 24 hours
		20,000	+	+	+	+	0	..	..	
		1,500	+	+	+	+	0			
		184	+	+	+	0				
		23	+	+	0					
		12	+	+	0					
		6	+	+	0					
		3	+	0						
1418 Serum agglut: 1:5 Bile agglut: <1:5	Mixed diet	160	+	+	+	+	+	+	+	Under paraffin oil
		23	+	+	+	+	+	+	+	
		12	+	+	+	+	+	+	+	
		2	+	+	+	+	+	+	+	Growth on 35th day
1181	Oats	500,000	+	+	+	+	0	..	..	Open tube; cotton stopper
		250,000	+	+	+	+	0			
		25,000	+	+	+	+	0			
		2,500	+	+	+	+	0			
		500	+	+	+	+	0			
		50	+	+	+	+	0			
		25	+	+	+	+	0			
		12	+	+	+	+	0			
		5	+	0						
1182	Cabbage	847,000	+	+	+	+	+	0	..	Open tube, cotton stopper
		423,500	+	+	+	+	+	0		
		42,350	+	+	+	+	+	0		
		4,235	+	+	+	+	+	0		
		847	+	+	0					
		84	+	0						
1200	Cabbage and $NaHCO_3$	500,000	+	+	+	+	+	0	..	Open tube, cotton stopper
		250,000	+	+	+	+	+	0		
		25,000	+	+	+	+	+	0		
		2,500	+	+	+	+	+	0		
		500	+	+	+	+	+	0		
		50	+	+	+	+	+	0		
		25	+	+	+	+	+	0		
		12	+	+	+	+	0			
		5	+	+	0					

As the germicidal action of hepatic duct bile is probably closely connected with the available bases in this secretion, an investigation of the behavior of this fluid obtained from an animal impoverished of these substances was important. A state of acidosis is readily produced in rabbits by feeding hydrochloric acid. The experiments were similar to those reported in the foregoing. The acid was fed to the rabbits operated on after the collection of the necessary control samples.

With the exception of rabbit 1031 b, no appreciable influence on the germicidal properties of hepatic duct bile of rabbits in a state of experimental acidosis could be noted. From this animal, after a total feeding of 0.72 gm. of HCl, a bile sample was obtained which sterilized itself of 2 strains of B. typhosus after 192 instead of 144 hours. The viability of B. dysenteriae was prolonged for an additional 24 hours. The minor differences recorded for the bile specimens of rabbits 32 and 1118 may be safely attributed to the method of testing the progress of sterilization and not to any material change in the bile itself. It is possible that more definite results would have been obtained if the animals had been kept on acid-forming diets and if the treatment with HCl could have been extended over a longer period. For practical purposes a continuous mild acidosis can probably be more satisfactorily obtained by fasting or by a predominant oat diet.

TABLE 4

THE PROGRESS OF STERILIZATION OF HEPATIC DUCT BILE OF RABBITS DILUTED WITH DISTILLD WATER

Four Rabbits. Diet: Mixed oats and carrots.

Changes Approximately Produced in Bile	P_H	Fistula Bile: 1:1000 to 1:10,000 Loopful of a 24-Hour Broth Culture								
		Typhoid K	Typhoid C	Typhoid J	Typhoid B	Paratyphoid A	B. Dysenteriae Shiga	Paradysenteriae Strain	B. coli	V. cholcrae
1 c c bile	8.4	72	84	96	72	96	24	24	132	228
1 part bile + 1 part distilled water	..	150	192	192	192	216	24	24	288	408
1 part bile + 2 parts distilled water	..	264	276	204	360	336	48	60	384	408
1 part bile + 3 parts distilled water	..	336	288	276	324	360	48	96	384	408
1 part bile + 9 parts distilled water	..	264	312	312	408	384	48	156	408	408
1 c c distilled water	..	24	24	24	24	24	24	36	132	72
1 c c peptone (0.1%) solution	..	400	408	408	408	408	108	240	408	408

DILUTION AND THE ADDITION OF SERUM TO HEPATIC DUCT BILE OF RABBITS

Observations reported above suggest that the germicidal properties of hepatic duct bile in all probability depend on other factors than merely progressively increasing alkalinity. Among the factors to be considered are the lack of nutritive material and the influence of H-ion concentration on the action of the bile salts. The first problem was investigated by progressively diluting bile samples or by adding heated sterile rabbit serum to strongly bactericidal bile specimens and inoculating each specimen with small amounts of typhoid bacilli.

In table 4 we summarized our results obtained on 4 hepatic duct bile samples collected from animals kept on various diets. It is quite evident that the progress of sterilization is retarded for from 3 to 5 days, when the bile specimen is diluted with distilled water in the proportion of 1:1. Moreover, progressive dilution produces a pronounced retardation in the antiseptic effect of one and the same bile sample. The distilled water used was doubly distilled and free from CO_2. B. typhosus and most of the other bacteria refuse to grow in it. These observations clearly demonstrate that the antiseptic properties of hepatic duct bile of the rabbit cannot be the result of a lack of nutritive substances. The H-ion concentration of the secretion exposed to air is only indirectly responsible for the gradual destruction of the inoculated micro-organisms. Even in a poorly buffered medium, such as the bile diluted 1:1 or even 1:2, the recordable change in the H-ion concentration is negligible and for practical purposes has no influence on the viability and growth of the inoculated bacteria. The actual hydrogen-ion concentration is decreased in a dilution of 1:9, and yet the various typhoid strains remained viable for more than 10 days in comparison to the short period of 4 days noted in the control tubes. It is our belief that dilution reduces the main germicidal substances, probably the effect of the bile salts. The time rate of lethal action is prolonged, irrespective of the low, unfavorable H-ion concentration. It is possible that the procedure of diluting bile induces other alterations, which we have not as yet investigated carefully. Even a profound change in the protein and salt content appears to be more favorable for the persistence of viable B. typhosus than the concentrated secretion supposedly rich in nutritive material.

In this connection it was naturally of interest to see how far the addition of nutritive material, for example serum, influences the rate of lethal action. According to Pies [23] and others, bile becomes regularly a good culture medium by the addition of organic matter, such as blood serum, pus or aleuronate. In table 5 the average time rate of lethal action of 8 hepatic duct biles diluted with serum on 2 strains of B. typhosus and 1 paradysentery strain is compared with that of undiluted and diluted bile. Contrary to the general belief expressed in the literature, fistula bile fortified by the addition of serum is not a medium in which B. typhosus can develop indefinitely. For example, the destruction in a specimen diluted with the serum in the proportion of 1:1 or 1:2 is identical with the one obtained for the same specimen diluted with distilled water. The prolongation of the viability of the organisms is therefore purely the result of a dilution of the antiseptic substances and not due to the addition of nutritive material or "vitamines." This conclusion is supported by the figures given in table 5; as has already been shown, the addition of small amounts of serum had no influence in its prolific effect. Our data support the conclusion of Pies that dilution with saline solution does not exert the same influence as distilled water. Further experiments are necessary before we feel justified in offering an explanation for this interesting observation.

The next factor or group of factors to be analyzed would be the bile acids and their disinfecting properties at various H-ion concentrations. Certain observations made on dog bile containing an excess of bile salts were so suggestive that we considered it advisable to conduct an extensive series of quantitative tests which will be reported in a separate paper.

TABLE 5

THE PROGRESS OF STERILIZATION OF HEPATIC BILE DILUTED WITH HEATED RABBIT SERUM

Rabbit	Diet	Amount of Heated Serum Added to Fresh Bile	P_{H^+}	Fistula Bile		
				Typhoid Kl 172	Typhoid K 120	Para-dysentery 310
8 Rabbits	Mixed Oats Cabbage and Carrots	1 c c bile	8.1	124	110	36
		1 c c serum	..	>240	>240	..
		1 c c bile + 1 c c serum	..	216	192	..
		1 c c bile + 0.5 c c serum	..	192	168	96
		1 c c bile + 0.25 c c serum	..	204	168	..
		1 c c bile + 0.2 c c serum	..	114	...	36
		1 c c bile + 0.1 c c serum	..	104	90	12
		1 c c bile + 0.05 c c serum	..	68	84	..
		0.95 c c bile + 0.05 c c serum	..	168	...	..
		0.75 c c bile + 0.25 c c serum	..	192	216	..
		0.5 c c bile + 0.5 c c serum	..	266	266	..
		1 c c bile + 0.5 c c saline	..	96	96	..
		1 c c bile + 0.5 c c distilled water	..	168	168	.
		0.5 c c bile + 0.5 c c distilled water	..	216	216	.

THE PROGRESSIVE STERILIZATION IN BILE DERIVED FROM HYPERCHOLESTEROLIZED RABBITS

Among the chemical components of the bile the various lipoids, soaps and cholesterol fractions deserve some consideration in the light of recent observations. Manfredi,[57] for example, reports that a concentration of cholesterin of 0.5% added to culture mediums will check typhoid and the paratyphoids, while B. coli will grow in concentrations up to 1%. It is worth noting that Bacmeister[74] and Exner and Heyrovsky[75] observed the decomposing effect of bacteria of the typhoid-paratyphoid group on bile, which frequently resulted in the precipitation of cholesterin. The experiments of Bassenge [55] with lecithins also deserve consideration. This worker demonstrated that these substances in 1% emulsions are destructive for typhoid bacilli in from 30 to 60 minutes. On the other hand, the growth-stimulating properties of unheated lecithin-containing mediums for B. anthracis and the tubercle bacillus is well known. Moreover, we recall the studies of Morato and Villanueva,[45] who demonstrated that cholesterol injections hasten the production of antibodies and agglutinins.

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[74] München. med. Wchnschr., 1908, pp. 211, 283 and 339.

[75] Arch. f. klin. Chir., 1908, 86, p. 609.

In order to determine the germicidal properties of lipoids and cholesterol we conducted a small series of tests with hepatic duct bile derived from hypercholesterolized rabbits. A fairly marked hypercholesterolemia was readily produced in these animals by feeding either lanolin (Merck's C. P.) or sheep brain or egg yolks. In the preceding paper data were recorded which demonstrated conclusively that it was possible to increase in rabbits the demonstrable blood cholesterol. Such a condition frequently led to a distinct precipitation of this substance in the gallbladder bile. The hepatic duct bile reached on one occasion only a concentration of 0.1% cholesterol, which according to the findings of Manfredi [57] is insufficient to exert a checking influence on B. typhosus. On the other hand, the gallbladder bile gave a concentration of 0.5%, despite which the growth and persistence of the viable B. typhosus were unhampered. The biles of cholesterolized rabbits were not any more germicidal than those of normal rabbits kept on various diets. Tests not detailed in this paper have confirmed in a general way these observations, and have shown that biles derived from fasting animals, which invariably have an increased cholesterol content (Rothschild [76]), differ little in their germicidal properties from those of normal animals.

Next in order for investigation would be the biliary mucin and the surface tension. A study of the latter is contemplated. An investigation of the antiseptic properties of biliary mucin appeared superfluous. Hepatic duct bile, which becomes antiseptic, is free from this substance, and cystic bile, which occasionally contains a considerable amount, is a good medium. This justifies the impression that the admixture of this secretion to hepatic duct bile of rabbits is in part responsible for the absence of germicidal properties.

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[76] Beitr. z. path. Anat. u. z. allg. Path., 1915, 66, p. 227.

A number of experiments were made by using ether or chloroform extracts of hepatic duct bile of rabbits. No difference in the influence on the growth of B. typhosus was observed in such biles in contrast to those untreated.

THE GROWTH OF B. TYPHOSUS IN HEPATIC DUCT BILE ENCLOSED IN COLLODIUM SACS IN THE PERITONEAL CAVITY OF THE RABBIT

It has been shown that hepatic duct bile of rabbit removed from the body becomes antiseptic when exposed to air. In an effort to rejuvenate the old antiseptic bile we returned it to the animal body in collodium sacs. This transformation of germicidal bile into a good medium was apparently accomplished in the following experiment.

Exper. 1.—A paralodium sac prepared over a glass guide with 4 holes sterilized in live steam for 1 hour was filled with 4 c c of hepatic duct bile (reaction P_H^+ 8.4) of rabbit 1182. This bile when tested immediately and when exposed to air destroyed 100 organisms per c c in 48 to 72 hours. The bile in the paralodium sac was seeded with an average of 50 typhoid bacilli (Strain K). The sac was carefully sealed and transferred to the peritoneal cavity of rabbit 1190. After 6 days had elapsed this animal was killed and carefully necropsied and cultures made. The tissues were found free from typhoid bacilli. The paralodium sac was covered by a thin layer of fibrin: there was no leakage of the membrane. The contents were pale greenish with a slightly brownish tinge and were perfectly clear. One c c contained 2.5 million viable B. typhosus. The reaction was P_H^+ 6.7.

It is evident that old, antiseptic hepatic duct bile in a paralodium sac, seeded with a small number of B. typhosus becomes a good culture medium for these organisms when placed in the peritoneal cavity of a rabbit. The most obvious change noted in this bile was an increase in the H-ion concentration; practically the optimum for B. typhosus had been attained. Naturally, this is only one of the changes which probably took place, and it would be erroneous to conclude that we reproduced in this experiment the conditions as they exist in the gallbladder. The observation is, however, of considerable interest, and further experimentation, particularly with longer exposure to the peritoneal fluid, may furnish important data on the influence of bile on B. typhosus and similar questions discussed in this paper.

THE RATE OF GROWTH AND THE SUBSEQUENT DISINFECTION IN GUINEA-PIG BILE

A series of quantitative tests with the hepatic bile of guinea-pigs that had been collected under oil, conducted in an identical manner to those reported for the rabbit, demonstrated, when seeded with B. typhosus, B. dysenteriae and Vibrio cholerae, a similar change in the rate of growth and in the subsequent development of antiseptic properties. One set of experiments is chosen in charts 3, 4, and 5, to illustrate the points of importance.

In contrast to our qualitative tests with bile collected unprotected in open tubes, in which most of the selected bacteria were destroyed in 48 to 96 hours, guinea-pig hepatic duct bile collected under oil is a good culture medium for the first 24 to 48 hours of incubation, and develops after this period germicidal properties for B. typhosus and B. dysenteriae. Bile samples kept exposed to the air or in a desiccator become antiseptic sooner for Vibrio cholerae than for B. typhosus and B. dysenteriae. It is, furthermore, shown that the same bile stratified with oil preserves the growth of Vibrio cholerae and also of B. typhosus, but does not prevent the development of germicidal substances for B. dysenteriae. The disappearance of viable dysentery bacilli occurs simultaneously in the tubes exposed to air or to alkali, or in the desiccator, as it does in the tubes stratified with oil. This event is fairly constant for a number of hepatic duct biles studied, and represents a noteworthy difference from the rabbit biles. It appears rather unlikely that under these conditions the development of germicidal properties is directly connected with a change in the reaction.

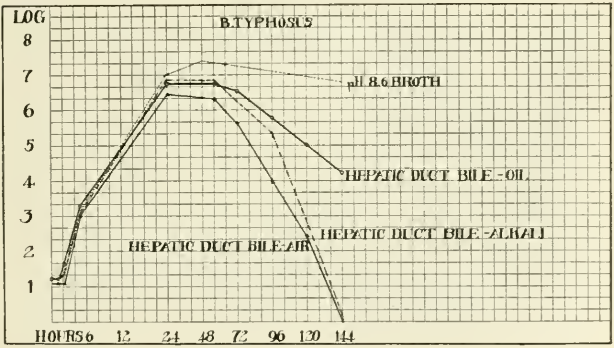

Chart 3.—Rate of growth of B. typhosus in the hepatic duct bile of guinea-pigs.

This suspicion is supported to a certain degree by the findings made on Vibrio cholerae. Chart 5 demonstrates the disappearance of viable spirilla in the tubes exposed to air and in the desiccator on the 96th and 144th hour, respectively. It is recalled that these organisms remain viable in rabbit bile for at least 288 hours, and in the desiccator even longer, at a period of the experiment when the alkalinity has probably reached its maximum (P_H^+ 9.2; titrable — 0.75). The persistence of bacteria rather sensitive to alkalies, like the Shiga dysentery organism in contrast to the alkalophilic Vibrio cholerae which disappears rapidly from guinea-pig bile, suggests the coexistence of other factors more potent than the change in the H-ion concentration as the agents responsible for the germicidal properties of hepatic duct bile exposed to air. Such substances develop rapidly on standing, when bile is collected in open tubes.

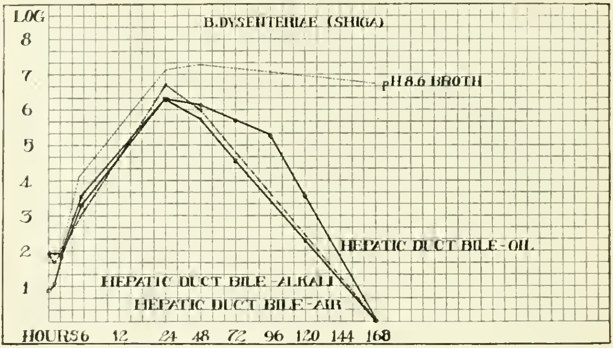

Chart 4.—Rate of growth of B. dysenteriae (Shiga) in the hepatic duct bile of guinea-pigs.

In another experiment bile was collected in open tubes and exposed to air for 4 hours before inoculation with B. typhosus. At the same time as a control a sample was stratified with paraffin oil. The sample exposed to air favored the growth for 5 to 8 hours; then germicidal substances produced a progressive disappearance of viable organisms. In 48 hours, sometimes in 24 to 36 hours, such a bile sample was completely sterilized. This experiment explains fully the results of Nichols, who collected the bile in open tubes and kept it at room temperature for more than 3 hours.

It has been repeatedly stated that dilution has little effect on the H-ion concentrations of bile. The tests under consideration are in every respect identical to those reported for rabbit bile with the exception that guinea-pig bile diluted with distilled water produces a more favorable medium than when diluted with saline. The distinct reciprocal relationship between dilution and viability of B. typhosus is also clearly demonstrated. The greater the dilution, the longer the persistence of viable organisms. Old and decidedly bactericidal biles, which destroy large numbers of B. typhosus in 6 hours, favored the growth and viability of these bacteria even in dilutions of 1:1 or 1:2. Differences in the H-ion concentration could not be recorded by our crude colorimetric method. The addition of 0.25 c c of sterile, heated guinea-pig serum prolonged the existence of viable bacteria in the proportion to the resulting dilution and not as would be expected in accordance with addition of nutritive material and buffers. Irrespective of all the changes brought about by these manipulations, the hepatic bile of guinea-pigs acquired antiseptic properties after a time which depended entirely on the degree of the dilution.

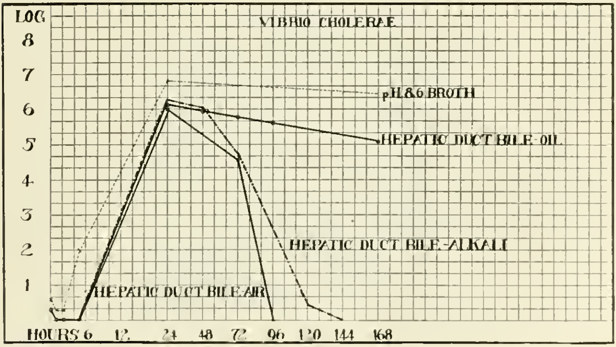

Chart 5.—Rate of growth of Vibrio cholerae in the hepatic duct bile of guinea-pigs.

Neutralization with lactic or metaphosphoric acid prolonged the persistence of viable B. typhosus for a few days. The results of these experiments were practically identical to those reported for the rabbit and the same interpretation can be applied to the guinea-pig's hepatic duct bile.

THE RATE OF GROWTH AND THE SUBSEQUENT DISINFECTION IN DOG BILE

The rate of growth and the lethal action of hepatic duct and of cystic bile procured from two dogs is shown in chart 6. Both animals showed a marked reflex in the secretion of the duct bile, and received either sodium taurocholate or ox bile during or before the collection of the bile specimens. Dog 17-170 secreted a nongermicidal bile for B. typhosus before the injection of sodium taurocholate. The pooled specimens collected after the injection were decidedly antiseptic as is clearly indicated by the curve in chart 6. A large number of typhoid bacilli was destroyed in less than 60 hours. Dog 17-220 was fed ox bile 4 hours previous to the operation and the collection of the bile specimens. B. typhosus grew fairly well for 30 to 40 hours. The bile, however, acquired antiseptic properties and after 120 hours was found to be sterile.

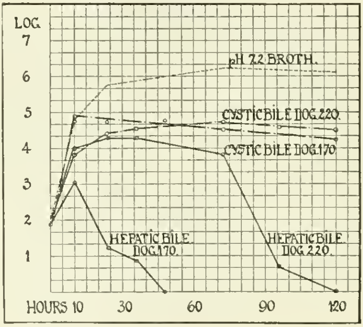

Chart 6.—Rate of growth in and lethal action of hepatic duct and cystic bile procured from two dogs.

Our observations which confirmed Ecker's,[19] prove the inhibitive and germicidal properties of bile salts on B. typhosus. In the course of test-tube experiments with bile salts added to suitable mediums of varying H-ion concentrations, it was noted that a P_H^+ over 8.0 markedly increased the bacteriostatic properties of bile salts in concentrations of less than 0.5%. A 1% solution of sodium taurocholate in a weak peptone salt solution at a P_H^+ of 8.4 was strictly germicidal. These observations have a direct bearing on the explanation of the germicidal properties of the two hepatic duct biles collected from dogs which were treated with sodium taurocholate in order to overcome the reflex inhibition of the bile flow. The familiar cholagogue action of sodium taurocholate given intravenously or by mouth, and the fact that from 40 to 90% of the introduced bile salts are eliminated in 4 to 6 hours (Foster, Hooper and Whipple [77]), immediately suggest a relationship between the increased amount of bile acids and the germicidal properties of hepatic duct bile. The absence of an antiseptic action of cystic bile is explained by the fact that the method of collecting the bile prevented the entrance of hepatic duct bile into this receptacle. These conclusions were confirmed by a quantitative analysis of the bile acids present in the hepatic duct bile of dog 17-170 and dog 17-220. We gratefully acknowledge the assistance received from the late Miss M. G. Foster, who made the determinations of taurocholic acid by the method developed by herself. The elimination of taurocholic acids were:

Dog 17-170 (weight 14.5 lbs.): Before the intravenous injection of 1 gm. of sodium taurocholate the hepatic duct bile contained 8 mg. of taurocholic acid per c c. In this sample B. typhosus grew for more than 10 days. After the intravenous inoculation of 2 gm. of bile salts the pooled bile specimen collected for a period of 5 hours (total 28.8 c c), contained 103 mg. per c c (or five times the maximum amount ordinarily found in hepatic duct bile or 1% of sodium taurocholate).

Dog 17-220 (Weight 45.25 lbs.): This dog was fed 400 c c of fresh ox bile by stomach tube 3 hours preceding the operation and the collection of bile samples. The pooled hepatic bile collected for a period of 4½ hours (total 106.9 c c). contained 63 mg. taurocholic acid or over 0.005% of sodium taurocholate.

The bile samples collected from the two dogs possessed, therefore, abnormal amounts of bile acids. In combination with the progressively increasing H-ion concentration, a strongly germicidal medium was produced. The gradual disappearance of viable bacteria in these bile samples is therefore readily explained. The absence of antiseptic properties in hepatic duct bile of some dogs treated with sodium taurocholate was due to a high H-ion concentration or perhaps to an absence of the cholagogue action of the administered bile salts or to both. It is obvious that the explanation offered may not be the only one, and that further investigations alone can furnish the answer to some of the questions which remain unsolved in this preliminary inquiry.

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[77] J. Biol. Chem., 1919, 38, pp. 379, 354.

THE RATE OF GROWTH AND THE SELECTIVE ANTISEPTIC ACTION IN FRESH AND OLD CYSTIC BILE OF THE OX

Fresh, unheated cystic bile of the ox inoculated with a large amount of typhoid bacilli still contained, after 10 days' incubation, viable bacteria. Fornet,[20] Ecker,[19] Pies [23] and others found certain bile antiseptic properties. Quantitative tests were made with fresh unsterilized gallbladder biles collected from beef animals in San Francisco during the months of March and April.

According to chart 7, comparatively small amounts of B. typhosus (strain K) in fresh unheated ox bile when stratified with oil grew freely, perhaps not as rapidly as in broth of a P_H^+ of 8.6. Even in the desiccator the development and persistence of viable organisms was fair. Decidedly antiseptic were the same bile specimens (5 c c) when kept exposed to ordinary laboratory air. A 24-hour period of active growth was followed by a progressive disappearance of living B. typhosus, and sterility was usually attained between the 72nd and 120th hour. The colorimetric titration gave always P_H^+ readings >8.6. The reaction changed slowly from a slightly, to a strongly, alkaline one, and regularly reached a lower H-ion concentration than the specimens kept in the desiccator, in which the absorption of ammonia was prevented. On the other hand, samples covered with oil showed occasion-

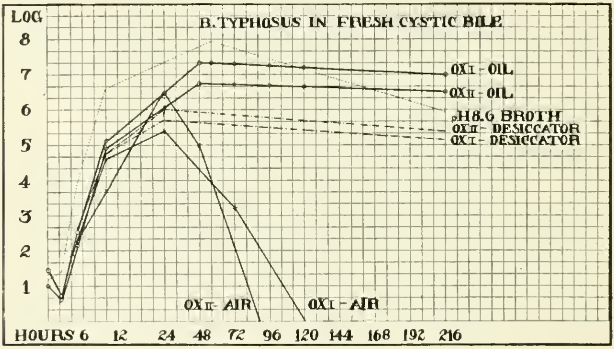

Chart 7.—Rate of growth of B. typhosus in fresh cystic bile of oxen.

ally a slight increase in the H-ion concentration from P_H^+ 8.0 to P_H^+ 7.6, probably due to the liberation of CO_2 by the cells or to lactic acid formation, the result of bacterial fermentation or destruction of cellular elements on standing. The difference in the behavior of ox bile in comparison to rabbit's and guinea-pig's bile was found in the tubes kept in a desiccator. We are inclined to explain this difference on the basis of a higher H-ion concentration. Furthermore, hepatic duct bile of rabbits has a different composition than cystic bile of oxen, and changes in the H-ion concentration may produce more potent germicidal forces in the former than in the latter. The varying composition of cystic ox bile intimately connected with the food of the animal is of greatest importance. It explains, in all probability, the varying results reported by different investigators.

The selective action of fresh unheated ox bile on Vibrio cholerae and B. dysenteriae Shiga was also studied. According to Schöbl, ox bile is a good culture medium for Vibrio cholerae. There may be a slight inhibitive action and the indication of a lag. The growth may be less heavy than in a plain salt-free peptone broth of a P_H^+ of 8.6. B. dysenteriae, however, cannot thrive in ox bile exposed to air; invariably 1500 organisms or less were destroyed in from 12 to 48 hours. This corresponds with the findings of Marbais.[78] Some specimens stratified with oil sterilized themselves of B. dysenteriae in 168 hours. In all three samples in which this took place, a shift of the H-ion concentration above P_H^+ 8.4 was noted and reached in the light of the observation of Cohen and Clark [79] the border of the P_H^+ zones. Reproduction and viability of B. dysenteriae Shiga is completely impaired. Tests with Flexner bacilli produced similar results. Occasionally, bile specimens were found which permitted an indefinite growth of these organisms, as Flu [12] has recorded for beef biles freely exposed to air.

Ox bile kept at room temperature exposed to diffuse light or in the ice-chest under oil or exposed to air and heated at 60 degrees for 30 minutes or autoclaved, produced a medium with a peculiar action on B. typhosus, which is illustrated by chart 8. In the experiments on which this chart was based, 5 c c samples of bile were inoculated with about 500 living B. typhosus. Antiseptic properties reduced this number materially in from 1 to 10 hours. This period of destruction of viable organisms varied with the different bile samples. The degree of germicidal action was occasionally so marked that a 0.1 c c specimen was found to be free from viable B. typhosus. If a small number of bacteria was seeded in such biles, a mortality of 100% or a complete sterilization might ensue, confirming Fornet [20] and Pies.[23] As a rule, however, the germicidal action on the part of the medium was followed by a pronounced more or less varying "lag" which in turn was succeeded by a gradual multiplication of the apparently seriously injured cells. Progressive proliferation of B. typhosus extended over 7 days, and not only reached, but sometimes surpassed the total number of viable organisms present in the broth control. This characteristic behavior of old heated cystic bile of the ox as a medium for B. typhosus is apparently not connected with the H-ion concentration of the secretion. Not only were the specimens acid to litmus and phenolphthalein, but the P_H^+ never decreased below 8.2. In the course of the experiment, probably as a result of the growth of B. typhosus, it even increased to P_H^+ 8.0 or even 7.6. From the standpoint of reaction, the ox bile became therefore more and more suitable. It is not unlikely that simultaneously a new strain was gradually generated with an acquired resistance to the antiseptic and bacteriostatic properties of the old bile. This new strain may in part be responsible for the slow but progressive

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[78] Compt. rend. Soc. Biol., 1918, 81, p. 1136.

[79] J. Bacteriol., 1919, 4, p. 409.

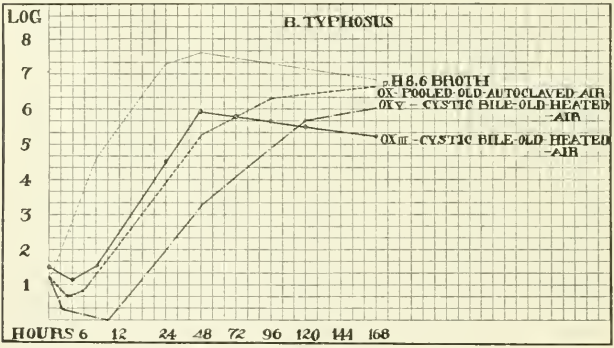

Chart 8.—Rate of growth of B. typhosus in bile of an ox.

proliferation after the 24th hour. A limited number of observations made by successive transfers of B. typhosus through biles of the same animal support this explanation.

As repeatedly emphasized, our study of ox bile was primarily undertaken to correlate the findings made with biles of other laboratory animals and to demonstrate conclusively that any bile on standing in a test tube undergoes changes which produce a germicidal, sometimes even a selective action, on a variety of bacterial types. Heating, filtering and other manipulations only enhance these properties and produce finally in vitro a medium of such complexity that it is impossible even to surmise how it acted in its natural environment, the gallbladder. The recognition of this fact, which does not apply to the study of bile alone but to many other body secretions, is frequently ignored by the majority of bacteriologists.

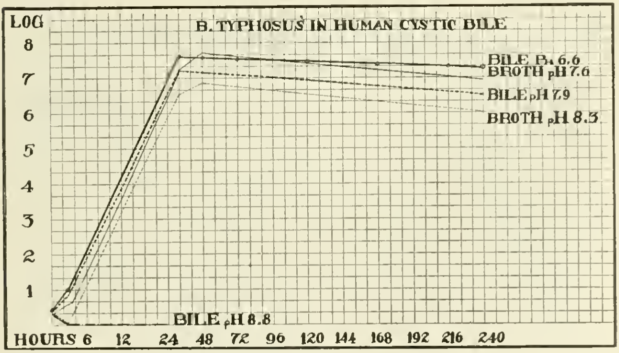

Chart 9.—Rate of growth of B. typhosus in human cystic bile.

THE RATE OF GROWTH OF B. TYPHOSUS AND VIBRIO CHOLERAE IN HUMAN CYSTIC BILE AT VARYING H-ION CONCENTRATIONS

Human cystic bile collected at necropsy or during laparotomies or cholecystectomies is, as a rule, a good medium for the development of a variety of bacteria which can remain viable for 60 days and longer. (Toida [27] and others.) Hepatic fistula bile proves a good medium for every micro-organism inoculated. Some investigators, Pies,[23] Mieczkowski,[32] Hirokowa,[30] and Toida[27] report, however, a marked degree of initial inhibition for a number of samples studied. We were unable to find such a specimen and can therefore not express an opinion on the nature of this bacteriostatic action. It is rather remote to suspect a change of reaction to be also responsible for this "lag," as was the case for the various other biles investigated. Our observations have demonstrated that the fairly alkaline or neutral human cystic bile changes slightly when standing exposed to air.

Fresh human bile was tested to compare its behavior with that in which the H-ion concentration had been previously artificially changed. The growth curves are shown in charts 9 and 10.

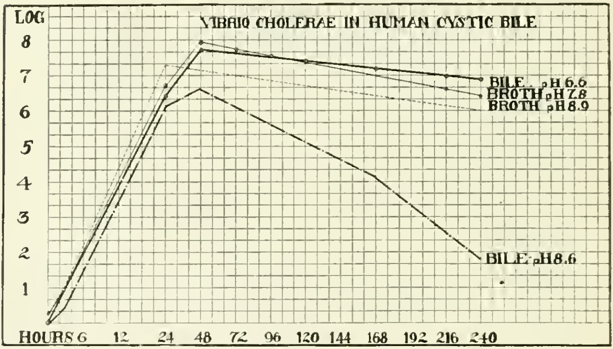

Chart 10. Rate of growth of Vibrio cholerae in human cystic bile.

It is evident that the proliferation of B. typhosus and Vibrio cholerae is only impaired or even completely suppressed at a H-ion concentration which in all probability never occurs in the human gallbladder. Human cystic bile must be considered a suitable medium for the viability of the two organisms; at least, in comparison with ox or rabbit bile the vigor of the bacterial growth is noteworthy. The sample chosen for the determination of the growth curves shown in the charts was derived from a gallbladder with a slight stasis as a result of fasting. The growth-favoring influence of starvation on the hepatic duct and cystic bile of rabbits has been previously mentioned. It was also pointed out that stasis favors gallbladder infection and therefore the carrier state. The growth curves presented here may therefore be of practical significance and suggest a possible connection between suitability of cystic bile as a medium for B. typhosus and for Vibrio cholerae; this may also explain the gallbladder carrier state. Variability in the composition, for example, changes in the proportion of taurocholic to glycocholic acid, and perhaps the reaction, influence the selective growth of different bile samples, as is well illustrated by the observations of Hirokowa,[30] Toida,[27] and others; but only a large series of tests will demonstrate beyond a doubt the actual importance and influence of the bile as such in the human typhoid carrier state.

SUMMARY

Hepatic duct bile of rabbits collected in open tubes and seeded with various pathogenic and nonpathogenic intestinal bacteria exhibits selective germicidal properties. The organisms of the typhoid-dysentery-paratyphoid group remain viable for 72 to 96 to 120 hours, and those of Vibrio cholerae and staphylococcus for over 7 to 10 days. A small number of bacteria is more readily destroyed than those of a heavy inoculum. Base-forming diets produce, on the average, bile specimens in which the time rate of lethal action is slightly shorter than in those procured from animals fed on acid-forming diets. True gallbladder bile specimens collected from the same animals and tested under the same experimental conditions fail to exhibit germicidal properties in the chosen time interval of 10 days.

Guinea-pigs' hepatic duct and cystic bile exhibits under identical experimental conditions greater germicidal action against the same number and the same type of bacteria than rabbit-liver bile. B. typhosus, B. paratyphosus, and B. dysenteriae are usually destroyed in from 24 to 48 hours; even Vibrio cholerae cannot survive a period of 120 hours. Fresh cystic bile of guinea-pigs, accidentally suffocated, was not germicidal for 400 hours.

Gallbladder bile of dogs kept on a mixed diet is apparently indifferent for a variety of bacteria; the inoculated organisms remain viable for over 10 days. Hepatic duct bile of the same animals treated with bile salts as cholagogues may be germicidal and destroy B. typhosus in from 24 to 120 hours.

Qualitatively at least the hepatic duct bile of cats, goats, rats, monkeys and man, as well as the gallbladder bile of oxen, sheep and pigs

favor the viability of B. typhosus. In such bile specimens inoculated with a large number of bacteria, living germs could be demonstrated in open tubes after 10 days of incubation at 37 C. Typhoid bacilli show a high degree of viability in human hepatic duct and cystic bile. Vibrio cholerae may be destroyed in 8 days. We failed to observe in one case of human biliary fistula after the institution of an extensive alkaline therapy, the development of germicidal properties against B. typhosus.

Nonpurulent gallbladder biles of typhoid-infected or carrier rabbits, guinea-pigs, monkeys and man behave either like hepatic duct bile specimens or like true cystic bile samples when removed from the viscus and exposed to air. Purulent specimens from rabbit or man, when incubated at 37 C., may show viable bacteria for over one month. The physical consistency, the cellular contents, and the duration of the carrier state govern the persistence of living B. typhosus in such specimens.

The germicidal properties of hepatic duct bile of rabbits and of guinea-pigs is not due to bacteriolysins or a "substance sensibilatrice." Such specimens never contain agglutinins. According to our observations, about 25% of the gallbladder bile specimens derived from laparotomized immune rabbits may contain agglutinins in a dilution of over 1:50. Recovered carriers may give clumping in dilutions above 1:100. The biles of guinea-pigs are always found to be free from agglutinins. One-half of the biliary secretions collected from "carrier" rabbits agglutinate B. typhosus in dilutions above 1:100. The degree of the inflammatory process and the stasis in the gallbladder are probably some of the factors which are conducive to the presence of agglutinins.

Quantitative tests with hepatic duct bile of rabbits collected under oil but tested in vitro exposed to air or to alkali have shown that B. typhosus grows, following a short lag, for at least 24 hours. However, in the next 24 to 48 hours, the bile acquires, probably on account of changes in the H-ion concentration (escape of CO_2, absorption of ammonia), antiseptic properties which lead, irrespective of the diet of the animal, to complete disinfection of the fluid in 96 hours. In hepatic duct bile stratified with paraffin oil and in cystic bile B. typhosus shows a high degree of viability. The Vibrio cholerae behaves similarly. These facts disprove, in our opinion, the theory that rabbit bile is germicidal in vivo.

Old, sterile bile kept at room temperature or in the ice-chest is germicidal for B. typhosus and B. dysenteriae Shiga after 24 hours' exposure at 37 C. Such bile specimens can be rejuvenated by saturation with CO_2 and subsequent stratification with oil. Neutralization of germicidal bile may produce temporarily, at least, a fluid in which a variety of bacteria can develop for from 1 to 8 days. The addition of strong acids alters the bile in many respects. Feeding or the injections of alkalies and acids, which produce a state of experimental alkalosis or acidosis, do not materially influence the germicidal properties of the hepatic duct bile of rabbits.

The H-ion concentration of the hepatic duct bile is only indirectly responsible for its antiseptic properties. Dilution of this secretion with distilled water in the proportion of 1:1 or even 1:2 fails to change the reaction but alters the concentration of the bile salts. It prolongs the persistence of viable bacteria for several days in comparison to the undiluted control tubes. Sterile serum acts in the same manner as distilled water. The growth-enhancing influence is in all probability not caused by the addition of nutritive material or "vitamines." The hepatic duct bile of hypercholesterolized rabbits develops germicidal properties after the same time interval as was observed for normal rabbits.

Antiseptic rabbit bile sealed in collodion sacs and placed in the peritoneal cavity of rabbits was found to be an excellent medium for the growth of B. typhosus.

Fistula bile of guinea-pigs collected under oil is a good medium for B. typhosus, Vibrio cholerae and B. dysenteriae. On standing exposed to air it changes its reaction and acquires germicidal properties which destroy the viable organisms in from 96 to 168 hours after inoculation and incubation at 37 C. One strain of an alkalophilic Vibrio cholerae was apparently more readily destroyed than several strains of B. typhosus and B. dysenteriae. The initial "lag" in fresh bile is slight and of short duration. Bile collected in open tubes or exposed for 4 to 24 hours to air becomes germicidal in a shorter time interval than fresh bile. The inoculated bacteria are destroyed in 24 to 48 hours or even less. Such biles, when diluted with distilled water or saline, favor the persistence of viable B. typhosus irrespective of the H-ion concentration. A distinct reciprocal relationship exists between dilution and viability of bacteria. B. typhosus will remain viable for over 10 days when the hepatic duct bile of the guinea-pig is diluted in the proportion of 1:9.

Fistula bile collected from dogs which received ox bile by mouth or sodium taurocholate intravenously in order to counteract the postoperative reflex inhibition on the biliary system, may contain more than 0.005% of taurocholic acid per c c. The salts of this acid are strongly antiseptic in an alkaline medium. As the hepatic duct bile of the dog also becomes alkaline (P_H^+ 8.2-8.4) on standing exposed to air, it is not surprising to observe the development of germicidal properties for B. typhosus in such specimens.

Fresh unheated sterile cystic ox bile develops, on incubation and exposure to air, germicidal properties. A certain number of the viable B. typhosus and B. dysenteriae are prevented from multiplication, and a large number of bacteria is actually destroyed in the first two hours after transference to the ox bile medium. This period is followed by a "lag" of varying length, which in turn is succeeded by a rapid proliferation. The maximum development is reached on the 12th or 48th hour for the specimen exposed to air. Progressive destruction, however, sterilizes these specimens in from 12 to 120 hours. The time required depends entirely on the bile specimen. The Vibrio cholerae is less seriously injured than typhoid and dysentery bacilli. Sterilization of the samples exposed to air or alkali has not been noted. Ox bile prevented by stratification with oil from giving off CO_2 or from absorbing ammonia in a desiccator does not become germicidal. Old, heated or unheated ox bile acts in a characteristic manner on B. typhosus. Immediately following the inoculation of the bile a marked destruction of viable organisms is recorded. This germicidal action may lead to complete sterilization of the bile, or is followed by a pronounced lag, which in turn is succeeded by a slow, but progressive, multiplication of the bacteria. The maximum growth may only be attained after 7 days, and the growth curve may at this time not only reach but even surpass the one of the broth control. This action of the bile is entirely independent of the reaction of the secretion. Our experiments confirm those of other workers, and they should prove conclusively that cooked ox bile is unsuitable for the primary isolation of B. typhosus from tissues or secretions. Mixed with peptone and glycerol it is, on account of its hemolytic properties, a good substratum for typhoid blood cultures.

The vigor of the bacterial growth in an average human cystic bile is noteworthy. Proliferation can be influenced by artificially reducing the H-ion concentration. Complete suspension of growth, however, only occurs at a P_H^+ which in all probability never occurs in the human body. The study of a large series of human cystic biles is advocated to determine the actual importance and influence of the secretion as such on the human typhoid carrier state. The observations reported in this paper have in our opinion demonstrated one outstanding fact, namely, a test-tube experiment may be simple and reveal a great deal of information, but it does not always tell the truth and may be most misleading to the pathologist.

THE INFLUENCE OF THE H-ION CONCENTRATION ON THE GROWTH OF B. TYPHOSUS IN MEDIUMS CONTAINING BILE OR BILE SALTS

EXPERIMENTAL TYPHOID-PARATYPHOID CARRIERS. VIII.

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In the preceding paper it was demonstrated that bile, particularly hepatic duct specimens, acquired germicidal properties when exposed to air. Comparative tests indicated that this effect was connected with a lowering of the H-ion concentration. However, it was not entirely evident in what manner this change in the reaction, alone or in combination with the chemical elements of the bile, had acted as the germicidal factor. A few experiments with bile specimens and bile salts at varying H-ion concentrations suggested themselves. These tests, primarily undertaken to study the rate of growth of B. typhosus and its generation time, furnished data which indicated that a low H-ion concentration inhibited the growth-stimulating properties of the bile and its salts and rendered such a medium germicidal.

With the exception of Meyerstein,[1] who states that the growth of B. coli and B. typhosus is abundant only when the mediums containing bile salts are neutral or slightly acid to litmus, recent reports on the subject of ox bile as a culture medium fail to mention the importance of the reaction. Ecker [2] and Salter [3] use neutral or slightly acid mediums which, according to the findings of Meyerstein, are the optimum reaction. Such a favorable reaction is, according to our tests, rarely encountered with the usual ox bile samples obtained in a fresh state from abattoirs, and sterilized while warm. It is quite obvious that the reports of various investigators dealing with the nutritive value of bile for bacteria must be more or less at variance on account of their failure to adjust the reaction of bile or bile salt mediums. Moreover, the proper-

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[1] Centralbl. f. Bakteriol., O., I, 1907, 44, p. 434.

[2] Jour. Infect. Dis., 1918, 22, p. 95.

[3] Ibid., 1919, 24, p. 260.

ties of sodium taurocholate or glycocholate and their decomposition products are not accurately known. On the other hand, it is not our intention to overemphasize the reaction of the bile salt mediums, but in connection with the experimental pathologic studies reported in the preceding papers, it appeared to us as one of the important factors among the many dealing with the so-called antiseptic effect of bile. In order to obtain striking contrasts, two reactions, namely, P_H 7.0 and P_H 8.2 to P_H 8.4 were chosen.

Technic.—One strain of B. typhosus (K) was used throughout the entire series of experiments. Transplants were kept on peptic digest agar. Previous to an experiment it was transferred for a few days to 1% ox bile salt-free veal broth. For each test a 18-24 hour old culture in this medium was used.

The salt-free veal broth was prepared in the same manner as stated in the second paper of this series. The 0.01% peptone solution, which was suggested by the work of Meyerstein, was made by dissolving 0.02% "Difco" peptone in distilled water and adding 20 c c of a phosphate mixture of known H-ion concentration to each 100 c c of medium. This mixture was distributed in 30 c c amounts in Pyrex Erlenmeyer flasks; sterile, filtered or unfiltered bile, or any other bile product to be studied was added and the total volume was made up to 50 c c. Sterilization was carried out in live steam on three consecutive days.

A 0.01% peptone solution, according to Meyerstein, is supposed to maintain the life of the organisms and yet not furnish enough nutritive material to permit a vigorous growth of B. typhosus. Hence any inhibitive or stimulating properties possessed by bile samples or their salts would be definitely demonstrated.

Several fresh samples of ox bile (12-14 cystic bile specimens) were filtered through paper and sterilized at 15 pounds' pressure. Sterilly collected hepatic duct bile samples of several rabbits were pooled and added without sterilization to the medium. Some experiments were also carried out with bile derived from a single gallbladder of the ox. Desiccated, "Difco" ox-bile was dissolved in distilled water and the concentrated solution added to the basic nutritive solution. This preparation is only slightly soluble in distilled water; a 1% solution forms an appreciable precipitate in the neutral medium. The latter was not filtered from the test medium, but care was taken to rotate the flasks thoroughly to insure even distribution of the sediment before samples for plating were removed. The purified bile salts were prepared by Drs. Foster and Hooper from dogs' or pigs' bile and have been tested by them in their studies on the metabolism of bile acids.

Fifty c c of medium were seeded with 0.5 c c of a 1:10,000 dilution of a young culture of B. typhosus in 1% ox bile salt-free veal broth. The flasks were warmed to 37 C. before inoculation and kept incubated at this temperature in an electrically controlled water bath. The determination of the number of organisms present at various periods of growth were made by plating in peptic digest agar (P_H 7.0). This medium was prepared in large quantities to insure uniformity in composition. Duplicate and often triplicate plates were poured with dilutions made in sterile salt solution. The plates were counted after 48

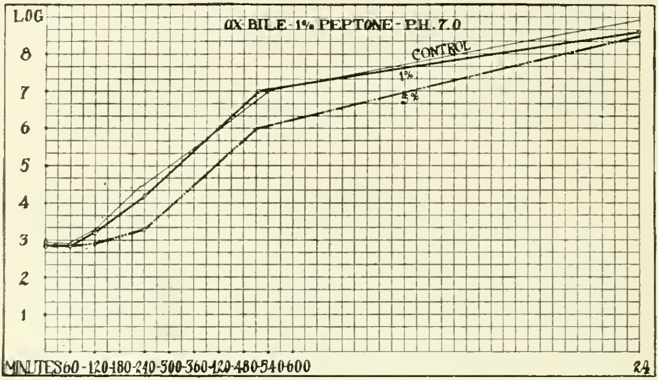

Chart 1.—Rate of growth of B. typhosus in filtered sterile ox bile in 1% peptone-phosphate solution, Ph 7.0.

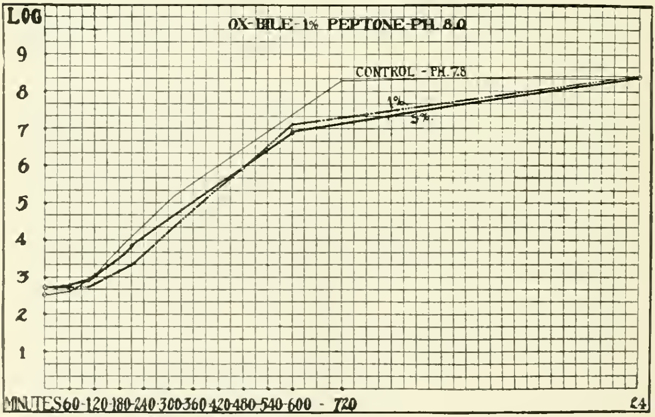

Chart 2.—Rate of growth of B. typhosus in filtered sterile ox bile in 1% peptone-phosphate solution, Ph 8.0.

hours' incubation at 37 C. Only plates which showed more than 25 or less than 250 colonies were considered accurate. Each experiment was repeated, and only the results in which no marked changes in the P_H reaction of the test medium occurred were noted. Representative data of such series are chosen for discussion of the individual experiments.

In the charts the logarithms of the viable organisms per 1 c c of medium are plotted as ordinates against time intervals (expressed in minutes) as abscissae.

Exper. 1.—Filtered sterile ox bile in 1% peptone-phosphate solution was seeded with B. typhosus and kept in water bath at 37 C.

This experiment clearly demonstrates that ox bile in low concentrations, namely 1 and 5% solutions, added to a suitable nutritive substratum, is distinctly inhibitive for B. typhosus. These observations confirm those recently published by Ecker. The growth curves of a 1% solution run below those of the control mediums throughout the entire period of the experiment. A 5% solution is decidedly depressant on the development of B. typhosus, a fact which is indicated by a distinct lag extending over 80 minutes. The final growth is, however, not materially influenced; the bile-curves show a tendency to reach the same level as the control-curve. Furthermore, the reaction of the medium does not materially alter the rate of growth, as is evidenced from the general behavior of the two curves. The minor differences in the character of the curves may be safely attributed to technical irregularities.

It was quite obvious that a 1% peptone solution was unsuitable to demonstrate conclusively the inhibitive or even the germicidal properties of bile. The available nutritive substances permitted a good initial growth, and the subsequent adaptation to the antagonistic forces of the bile produced the results clearly demonstrated in the curves. Identical observations were made by Pies.[4] The suggestion of Meyerstein to use a 0.01% peptone solution was therefore followed. It was unfortunate that our experiments, which were intended to retain the initial reaction constant, necessitated the addition of a phosphate mixture. A peptone-phosphate solution is, in contrast to Meyerstein's Witte's peptone solution, a fair culture medium, as the figures in the table clearly demonstrate.

It is evident from the table that the claims of Meyerstein were not confirmed. In several tests a distinct increase of the inoculated B. typhosus took place after 24 hours' incubation. The multiplication was not as marked as in a "Difco" peptone solution, or in the same solution buffered with phosphates. The phosphate mixture at a P_H 7.0, kept B. typhosus alive or stimulated a slight growth. It is, therefore, advisable to test in such a solution the substances which are suspected to exert an inhibitive or germicidal effect on bacteria. For the graphic demonstration of the influence of the reaction on the action of bile salts a 0.01% "Difco" peptone solution with phosphates reproduced the nutritive value of the bile more accurately than the phosphate mixture alone. This conclusion was justified by the numerous successful tests to be reported.

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[4] Arch. f. Hyg., 1907, 62, p. 107.

Growth of B. Typhosus in Peptone and Peptone-Phosphate Mixtures Ph 7.0

	Number of Organisms Immediately After Inoculation per C c	Number of Organisms After 24 Hours per C c
Phosphate mixture alone..........................	410	860
Difco peptone, 0.01% solution....................	1,600	13,000,000
Difco peptone, 0.01% solution + phosphate mixture	1,600	34,000,000
Witte's peptone, 0.01% solution..................	1,600	1,350,000
Witte's peptone, 0.01% solution + phosphate mixture	1,680	1,560,000

Exper. 2.—Filtered sterile ox bile in a 0.01 "Difco" peptone-phosphate solution, P_H 7.0 and P_H 8.2, was seeded with B. typhosus. Incubation occurred at 37 C. Four tests were made.

At a reaction of P_H 7.0, ox bile in quantities of 1% or less was decidedly stimulating for B. typhosus. Even the usual lag was entirely absent, and the figures obtained for the final growth exceeded the one for the control flask. Five, 10 and 30% additions of ox bile were not only inhibitive, as indicated by the prolonged flag, but were also depressing the growth curve in general. Attention is called to the inhibition of the growth for at least 5 hours in the 10 and 30% ox bile-peptone solutions. Moreover, in contrast to the 1% peptone solution, the development of B. typhosus in a 0.01% peptone medium was slow but progressive, and the curve failed to show the steep rise in the first 8 hours so clearly noted on charts 1 and 2.

At a P_H 8.2, ox bile, even in concentrations below 1%, was distinctly inhibitive. The initial lag for 10 and 30% solutions was extended over a period of from 8 to 9 hours. Furthermore, the final growth as a whole was considerably below the one noted for the medium of a P_H 7.0. In many respects the general character of the 30% ox bile curve corresponded to the one established in the preceding paper (chart 8) for fresh unsterilized ox bile of a reaction of P_H 8.2-8.6. These findings

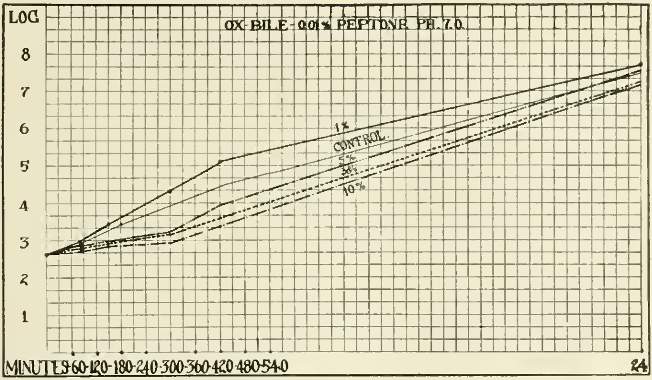

Chart 3.—Rate of growth of B. typhosus in filtered sterile ox bile in 0.01% "Difco" peptone-phosphate solution, PH 7.0.

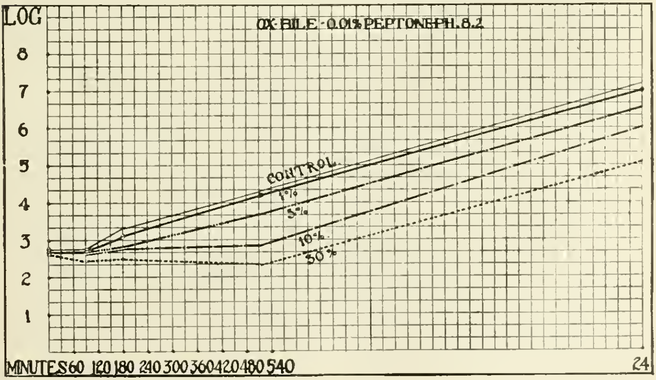

Chart 4.—Rate of growth of B. typhosus in filtered sterile ox bile in 0.01% "Difco" peptone-phosphate solution, PH 8.2.

demonstrated that ox bile at a P_H 8.2 inhibits a large proportion of the viable cells.

The nature of the inhibitive substances in ox bile is for the present omitted from consideration. Whether the specific stimulating effect of this secretion in small concentrations at a P_H 7.0 is merely the result of additional nutritive material or due to certain definite substances, is difficult to say. It is recalled that Salter found bile salts in small quantities to be growth promoting for B. coli. Bile in low concentrations can therefore act in a similar manner on all members of the colon typhoid-paratyphoid group.

Exper. 3.—"Bacto" desiccated ox bile in 0.01% "Difco" peptone-phosphate solution, P_H 7.0 and 8.2, was seeded with B. typhosus. Incubation ocurred at 37 C. Two experiments were made.

The influence of the reaction on the effect of bile salts as culture mediums for B. typhosus was well illustrated by this experiment. Thus it was shown that neutrality supported the growth producing properties of a low concentration of bile salts, while a strongly alkaline reaction rendered the same substances not only inhibitive, but germicidal. At P_H 7.0 a 30% "bacto"-bile salt medium was slightly inhibitive and a 0.5 and a 1% solution were stimulative. The curve for a 2% bile salt medium was not shown in the chart. It covered the one for the control medium.

At 'a P_H of 8.2 a 1%, even a 0.2%, solution of desiccated bile was germicidal for B. typhosus. The rate of lethal action was shortened by the concentration of the medium in biliary elements or, in other words, the more concentrated the fluid, the greater was its effect on the viable cells. It was furthermore shown that the germicidal action did not manifest itself for at least 3 hours in the 0.2 and 0.5% bile salt medium. There were indications that for this time period the inoculated B. typhosus not only remained viable, but proliferated to a slight degree. This particular phenomenon was not absolutely constant and certain unknown factors, possibly similar to those noted by Cohen and Clark, produced slight variations. The curves shown in charts 5 and 6 represent the general behavior of B. typhosus in bile salt mediums at different H-ion concentrations, and find their analogues in some of the charts made from determinations on fresh, undiluted animal bile, discussed in the preceding paper.

Exper. 4.—Sterile hepatic duct bile of rabbits in 0.01% peptone-phosphate solution was seeded and incubated as usual.

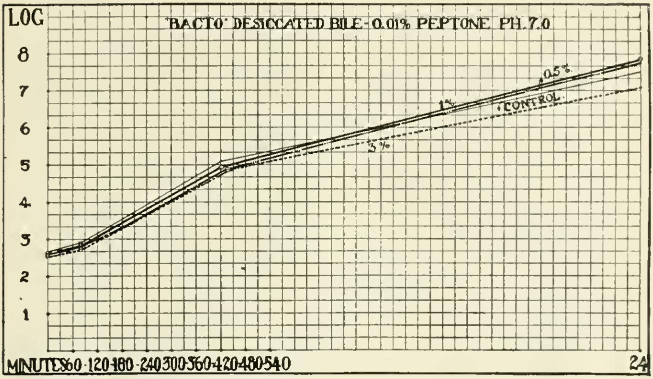

Chart 5.—Rate of growth of B. typhosus in "bacto" desiccated ox bile peptone-phosphate solution, Pʜ 7.0.

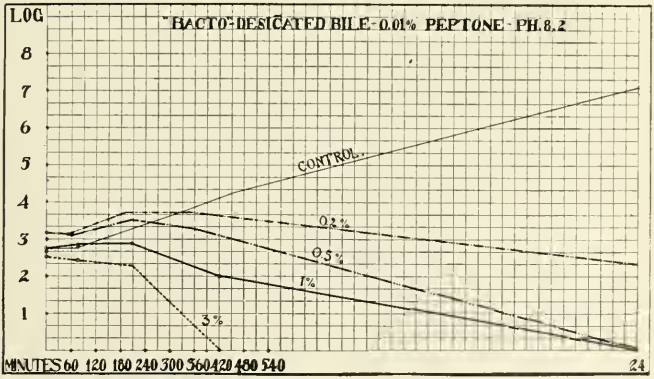

Chart 6.—Rate of growth of B. typhosus in "bacto" desiccated ox bile peptone-phosphate solution, Pʜ 8.2.

The curves presented in charts 7 and 8 confirmed our previous observations with undiluted hepatic duct bile of the rabbit exposed to air for several days. However, the data revealed some interesting properties which were not evident in the tests conducted with undiluted bile. Rabbit bile was not only less inhibitive than ox bile in a neutral medium, but must be considered stimulating and growth enhancing. Additions of bile (not exceeding 10%) invariably produced better development of B. typhosus than a peptone-phosphate mixture alone. The paradoxical behavior of the 5 and 10% solutions of rabbit bile was constant in the 2 experiments conducted. For the present we are unable to offer an explanation for the observations, but desire to call attention to a similar observation recorded by Meyerstein. In the course of some tests with purified crystallized ox bile, this worker noted that B. typhosus and B. coli grew well in a 10% solution of a mixture of sodium taurocholate and sodium glycocholate (40 and 60%), while lower concentrations (1 to 5%) completely inhibited proliferation of these organisms. He states that this action of the bile salts was not recorded with the desired regularity to permit a final conclusion. In the light of the experiments conducted with pure bile salts, it is suspected that these paradoxic results are due to the glycocholic acid or its salts. Further tests are, however, necessary to prove this contention in a conclusive manner.

The general tendency of the growth curve at P_H 7.0 also indicated that the typhoid bacillus adapted itself more readily to the elements contained in the rabbit bile than to those of ox bile. The initial lag was comparatively short and between the 2nd and 6th hour the proliferation was very active in the 5 and 10% solutions. The final growth at the end of 24 hours, however, did not materially exceed the one reported for ox bile.

An alkaline reaction of P_H 8.4 affected the inoculated bacteria in a manner already known. In the 2 experiments the results differed somewhat from each other, but the curves shown in chart 8 present the average behavior of B. typhosus in rabbit bile at P_H 8.4. Moreover, the 5% solution acted paradoxically, while the 1 and 10% mediums followed the course to be anticipated. Both the 5 and 10% bile-peptone solutions exhibited distinct germicidal properties in the initial 5 hours, which was followed in the 5% solution by a slow, but progressive proliferation of the remaining viable cells. On the other hand, the 10% solution continued to destroy the inoculated germs until com-

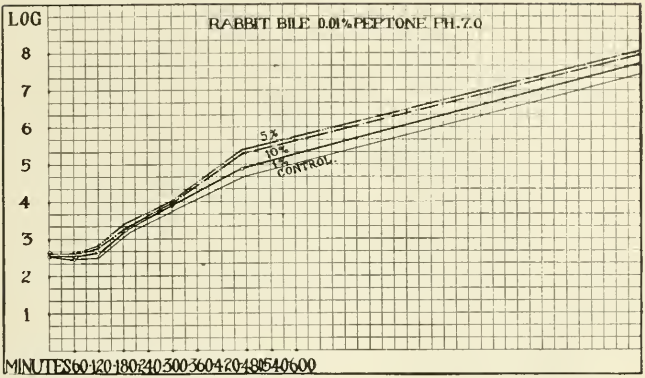

Chart 7.—Rate of Growth of B. typhosus in sterile hepatic duct bile of rabbits in 0.01% peptone-phosphate solution, PH 7.0.

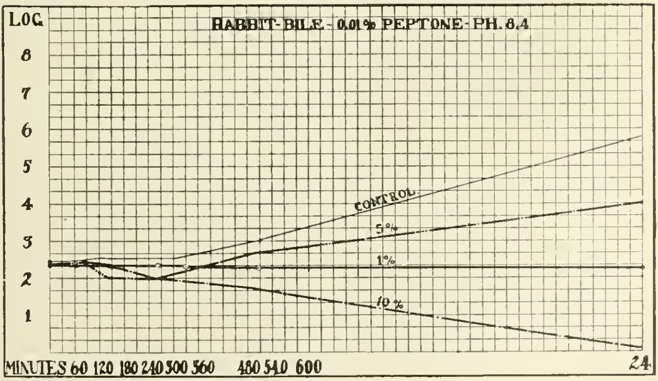

Chart 8.—Rate of growth of B. typhosus in sterile hepatic duct bile of rabbits in 0.01% peptone-phosphate solution, PH 8.4.

plete sterilization resulted in the 24th hour. The 1% solution exhibited a distinct bacteriostatic action. The available figures indicate that typhoid bacilli either remained alive or were slightly reduced in numbers. At P_H 8.4 rabbit hepatic duct bile added in varying amounts to peptone-phosphate solutions may therefore exhibit three different properties: it maintains growth, it is bacteriostatic, or it is germicidal. The various properties ascribed to animal bile by numerous investigators can obviously be recorded on one and the same specimen. The nature of the inhibitive substances has been considered in the preceding paper. It was concluded that in all probability the bile salts deserve a more detailed investigation. With this conception in mind a series of tests were conducted with pure bile salts.

Exper. 5.—Sodium taurocholate and sodium glycocholate in a 0.01% peptone-phosphate solution were seeded and incubated as usual. Three experiments were made.

A superficial inspection of the curves revealed the same tendency in the rate of growth of B. typhosus in pure bile salt mediums as already noted and discussed for the fresh or the desiccated ox bile. The growth of B. typhosus was excellent at P_H 7.0, but it was inhibited at P_H 8.4. Moreover, distinct differences existed between the peptone solution containing sodium glycocholate and those prepared with sodium taurocholate. At neutrality sodium glycocholate in 0.5% and 1% solutions was bacteriostatic for 3 hours, but at the end of 24 hours the number of viable cells exceeded those of the control flask. A somewhat similar effect was noted for the taurocholates. From the available data, however, it was impossible to state whether sodium glycocholate was more stimulative at P_H 7.0 than the sodium taurocholate. In comparison with the results obtained at a low H-ion concentration the observation deserves recognition and will repay additional investigation.

The outstanding feature of the action of purified bile salts at P_H 8.2 was the strongly germicidal effect of sodium glycocholate in 0.5% concentration, the inhibitive and slightly antiseptic effect of 1% sodium taurocholate, and the growth-depressing properties of the same salt in 0.5% solutions. It was quite evident that in alkaline mediums glycocholates were considerably more germicidal than the taurocholates, a fact which has hitherto not been emphasized. Meyerstein[5] noted the absence of growth of B. typhosus in a 0.01% Witte's peptone solution

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[5] Centrlbl. f. Bakteriol., I, O., 1904, 44, p. 138.

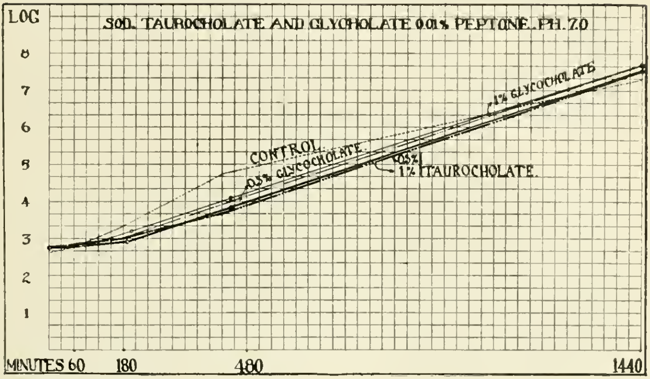

Chart 9.—Rate of growth of B. typhosus in sodium taurocholate and glycocholate in a 0.01% peptone-phosphate solution, Ph 7.0.

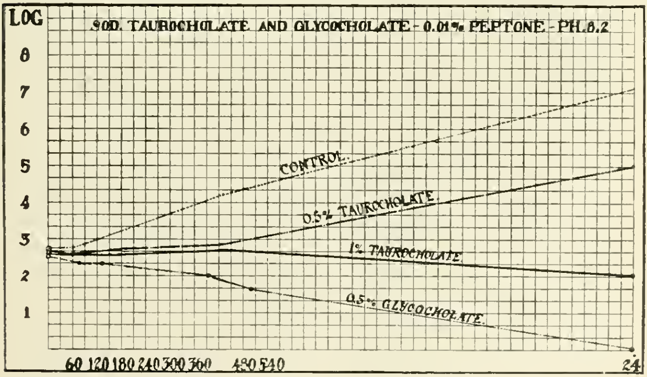

Chart 10.—Rate of growth of B. typhosus in sodium taurocholate and glycocholate in a 0.01% peptone-phosphate solution, Ph 8.2.

containing 5% sodium glycocholate, while Dünschman[6] apparently made a similar observation, which prompted him to write the following sentence: "le glycocholate, de son côté agit plutôt à la façon d'un antiseptique."

Even the growth maintaining 0.5% sodium taurocholate-peptone solution was not indifferent to the typhoid bacillus: an 8-hour lag precedes the slow but progressive growth.

DISCUSSION

It is evident from the foregoing data that the rate of growth and the action of the bile and the bile salts of various animals on B. typhosus in simple mediums is materially influenced by the reaction. At neutrality small amounts of ox bile, "Bacto" desiccated bile, sodium taurocholate and glycocholates and fairly large amounts of hepatic duct bile of the rabbit are distinctly stimulating. These results are definitely demonstrable when a simple 0.01% peptone-phosphate solution is used as a basic substratum. As a rule, bile concentrations of over 1% are distinctly inhibitive at a P_H 7.0. Rabbit bile in this respect offers an exception for the following reason: It is technically impossible to obtain a sufficient amount of cystic bile to conduct a well-controlled series of tests. The hepatic duct bile employed is less concentrated and contains about $1/10$ of the organic and inorganic elements ordinarily encountered in the gallbladder bile of cattle. It is also possible that rabbit bile is an exceptionally good medium for B. typhosus at a neutral or slightly alkaline reaction. Our observations made in vivo and in vitro with cystic bile of these animals and reported in the preceding papers lend some support to this contention.

The nature of the stimulating action has not been investigated. It may be that the bile or bile salts supply only additional food material to a comparatively poor nutritive substratum. The bile and bile salts may contain some of the mysterious substances classified with the so-called "vitamines" that play an important rôle in animal metabolism. Viewed from this standpoint the bacteriologic aspect of bile offers an unlimited number of experimental possibilities.

In an alkaline medium (P_H 8.2 to 8.4) the bile specimens or their salts invariably cause distinct inhibition in concentrations below 0.5%. The addition of comparatively large amounts of the same bile or biliary salts renders the medium not only inhibitive, but directly germicidal.

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[6] Ann. de l'Inst. Pasteur, 1909, 23, p. 48.

In all such mediums the lag is increased, and there may be a distinct bacteriostatic effect for from 5 to 10 hours. A slight initial growth may be followed by a slow, but progressive, destruction of the viable cells. In ox bile the prolonged lag may be replaced by an active growth leading after 24 hours to a number of viable cells nearly as great as the one recorded in the bile-free control tube. This phenomenon is probably the result of adaptation of B. typhosus to the antagonistic forces of the alkaline-bile mediums. It is possible that the inhibitive substances, as for example the bile salts, are decomposed as a result of the growth of B. typhosus (Exner and Heyrovsky) and transformed into growth-enhancing food elements. Our experiments are not sufficiently numerous, nor have they been planned with this question in mind. The data at our disposal permit one conclusion, namely, that the bile salts, and particularly the glycocholates, are the substances which inhibit or diminish the viable cells of B. typhosus in weak peptone solutions. Furthermore, the interesting and noteworthy fact is recorded that the bile salts in the concentration usually present in normal bile exert their germicidal properties when the medium is alkaline. In searching for an analogous condition, the result of studies of several investigators are recalled, namely, certain dyestuffs are more potent in a medium with a reaction in the alkaline range than in one at or near neutrality. Prowazeck[7] and Traube[8] found that sodium carbonate accentuates the toxicity of methylene blue or of crystal violet. Browning, Gulbranson and Kennway[9] also obtained remarkable results in the sterilizing effect of diaminoacricidine-methyl-chloride by changing the P_H of the medium from 4.0 to 11.0. Neither of these workers offers a conclusive explanation for this phenomenon, but it is not unlikely that the view of Traube, who found that sodium carbonate produced changes in the surface tension of the dye solutions, is applicable to the bile salts mediums. In this connection the observations of Larson, Cantwell and Hartzell[10] with pneumococci should be mentioned. Bile reduces the surface tension of fluids and, according to the foregoing writers, favors the disintegration of pneumococci. How far our findings are analogous to those just stated must be determined by further tests. One fact is certain: Bile salts are more readily soluble in alkaline solution than in neutral or slightly acid mediums.

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[7] Arch. f. Protist., 1910, 18, p. 221.

[8] Biochem. Ztschr., 1912, 43, p. 496.

[9] Jour. Path. & Bacteriol., 1919, 23, p. 106.

[10] Jour. Infect. Dis., 1919, 25, p. 45.

For the present the selective germicidal action of glycocholates in alkaline mediums cannot be explained. An observation of considerable practical importance is that bile and bile salts derived from various animals contain varying amounts of this acid. According to Hammarsten,[11] rabbit bile and some samples of ox bile possess exclusively glycocholates; while dog bile is stated to be deprived of this substance. Variation in the cultural properties of bile samples must be ascribed to the composition of the secretion, and not purely to the reaction, as is the case in the observations of Nichols. Chemical analyses of hepatic duct bile of rabbits are not available for comparison, but it is not unlikely that similar unexplicable fluctuation in the bile acid content may occur, as has been so clearly demonstrated by Foster, Hooper and Whipple [12] for the dog. Future bacteriologic studies on bile should therefore appreciate not only the variability of the reaction, but of the composition as well.

In all probability the antiseptic action of glycocholates or taurocholates does not occur in the animal body. A reaction conducive to the development of germicidal properties is found only in the test tube exposed to air, and not in the gallbladder in vivo. This point has been treated in detail in the preceding papers.

As far as the recorded observations have some practical bearing on the use of bile or bile salt mediums, it can be stated that such additions to nutritive mediums will be advantageous only in neutral or slightly acid substratums, and when the concentration does not exceed 1% fresh or 0.5-1% desiccated ox bile. The value of bile additions should never be over-estimated, primarily on account of the comparatively slight stimulating, selective effect on B. typhosus, secondarily on account of the production of a distinct lag, which is provoked by fairly small amounts of bile salts. The recommendation of Tonney, Caldwell and Griffin [13] in disregarding lactose bile for the isolation of B. typhosus from the stool is fully justified in the light of our findings.

CONCLUSIONS

Bile of oxen, hepatic duct bile of rabbits, bacto "desiccated ox bile," sodium glycocholate and taurocholate in 1% concentration in a 0.01% "Difco" peptone-phosphate solution at a P_H 7.0 are growth-enhancing for B. typhosus, while greater amounts, such as 3 to 30%, greatly inhibit proliferation. At P_H 8.4 the same bile specimens or their salts acquire either inhibitive, bacteriostatic or germicidal properties. The more concentrated the mediums are in biliary salts, the greater is their effect on the viable cells. Even small amounts of bile salts, such as 0.5%, destroy the inoculated bacteria in 24 hours. At P_H 8.4 glycocholates are more antiseptic than taurocholates, while the same salts in the same concentration may be stimulative at a P_H 7.0.

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[11] Ergebn. d. Physiologie, 1905, 4, p. 1.

[12] Jour. Biol. Chem., 1919, 38, p. 379.

[13] Jour. Infect. Dis., 1916, 18, p. 239.

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THE PATHOGENICITY OF B. ABORTUS AND B. MELITENSIS FOR MONKEYS

STUDIES ON THE GENUS BRUCELLA NOV. GEN. III.

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Recent observations made by A. C. Evans,[1] by Meyer and Fleischner, Shaw and Feusier[2] and by Zeller[3] have proven that B. abortus and B. melitensis are morphologically, biochemically and serologically closely related. In order to demonstrate conclusively the relationship of the two bacteria, it becomes necessary to conduct infection experiments on animals. In several series on guinea-pigs, it has been demonstrated that the lesions produced in the spleen, the lymph nodes, etc., following an intratesticular inoculation of certain B. melitensis strains, are indistinguishable from those produced by B. abortus. As the majority of the experiments by early observers on B. melitensis has been carried out on the monkey, an animal which, as originally shown by Bruce[4] and afterward by Hughes,[5] can be easily and certainly infected, it has seemed advisable to make similar experiments with B. abortus. The various reports of the Commission for the Investigation of Mediterranean Fever record numerous experiments dealing with infections produced in monkeys by subcutaneous, intramuscular and intravenous inoculations. Even the feeding of a single sample of infected goat's milk to 28 monkeys caused in 26 of them a disease which was very similar to that seen in man. It is stated that, as a rule, a B. melitensis infection of a monkey can be judged by the animal's temperature, which may be intermittent or remittent in character, by the appearance of specific agglutinins in the blood serum, and by slow, but definite loss in body weight.

As soon as the close relationship of B. abortus to B. melintensis had been established, it was manifestly important to determine experi-

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[1] Jour. Infect. Dis., 1918, 22, p. 580.

[2] Proc. Soc. Exper. Biol and Med., 1919, 16, p. 151; Jour. Infect. Dis., 1920, 27, pp. 173 and 185.

[3] Berl. tierarztlich. Wchnschr., 1920, 36, p. 345.

[4] Ann. de l'Instit. Pasteur, 1893, 7, p. 289.

[5] Lancet, 1892, 2, p. 1265.

mentally the following question: Can B. abortus produce in the monkey a similar syndrome as B. melitensis? Careful review of the work that had been done with B. abortus showed that only one investigator used monkeys. Marshal Fabyean,[6] in his studies on the persistence of this organism in the tissues of inoculated animals, infected two monkeys and was able to recover B. abortus 12 and 13 weeks, respectively, after the inoculation. He did not state the method used in inoculating the animals and gave no additional observations as to the results.

Aside from reproducing an undulant fever-like disease by direct inoculation of B. abortus, it was obviously important to determine carefully the possibility of causing an infection by feeding either cultures or goats milk containing the bacteria in small or large numbers. These experiments led to a consideration of the so-called "virulence" of B. abortus and resulted in a number of similar experiments with strains of different origin. It seemed also possible that more definite knowledge might be obtained concerning the pathogenicity of B. abortus for children and adults, if a study of feeding infections in monkeys were undertaken. The experiments reported here, therefore, were planned to supply a number of facts, which would assist in answering the question in a somewhat more intelligent manner than had hitherto been possible.

In order to be familiar with the temperature reactions, and the symptoms produced in monkeys by the organisms of malta fever, a number of infection experiments were made with certain interesting strains of B. melitensis. These cultures had been found to be pathogenic for guinea-pigs, and quite frequently they could not be differentiated from those of true abortion bacilli.

METHODS

Most of the experiments were made on young monkeys and if possible on one species, namely, Pithecus syrichta, imported from India. All were fresh stock and were brought directly to the laboratory on their arrival by steamer in San Francisco. On two occasions two old stock animals, a Macacus cynomolgus and a Cercopithecus philippinensis were also used. The animals were kept in single cages and special care was taken to avoid cross infection by contaminated food, urine, etc. This precaution was particularly necessary in connection with the B. melitensis experiments.

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[6] Jour. Med. Res., 1913, 28, p. 82.

The organisms used for the tests were grown on glycerol peptic digest agar slants (P_H 7.0) at 37 C. for 2-7 days. The monkeys were fed by either smearing the bacterial growth on carrots or by suspending the cultural growth in 50-100 c c of milk. The latter was offered to the starved animals in small pannikins. As a rule, the monkeys drank the milk without wasting more than 20-30 c c. Blood stream inoculations were made by way of the jugular or saphenous vein.

The methods of study after inoculation or feeding consisted in the observation and record of clinical symptoms, the taking of morning temperature (rectal), repeated agglutination tests with the blood serum, and hemoglobin determinations. Formalinized suspensions of B. abortus and B. melitensis as specified in previous papers were used as antigens for the agglutination tests. Necropsies with bacteriologic examinations were made on all animals that died or were killed. Cultures of the tissues were made on blood agar plates, glycerol peptic digest agar slants and glycerol veal infusion broth. The strains isolated were identified by agglutination and absorption tests, as detailed in paper II of this series.

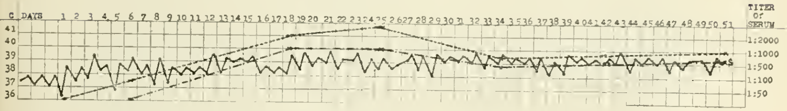

Chart 1 (Monkey 2).—Intravenous inoculation of B. abortus.

EXPERIMENTAL DATA

Infections Produced by the Intravenous Inoculations of B. abortus.—Three normal monkeys were injected intravenously with typical abortion bacilli in amounts ranging from 2-16 billion of a glycerol peptic digest agar culture. The following protocols detail the various observations.

Exper. 1.—*Monkey* 2.—Pithecus syrichta, female, weight 1,875 gm. March 18, to April 3, 1919, well and active; serum repeatedly examined for B. abortus and B. melitensis agglutinins with negative results. April 3, 3:00 p. m., inoculated intravenously in left jugular vein ⅕ slant of a 48-hour old peptic digest agar slant—2 billion of B. abortus 80 (origin of culture—certified milk—guinea-pig passage, 2½ years old); bled from the right ear 15 minutes after injection.

Blood smeared on agar slant gave profuse growth of B. abortus. The temperature during the period of observation of 51 days was always above 39 C. (chart 1).

April 24, the animal developed diarrhea which lasted for 2 days. Blood cultures on the sixth and eighteenth day remained sterile. The blood gave the following agglutination reactions:

April 9, or 6 days after injection—B. abortus 80=1:80; B. melitensis 11=0.

April 21, or 18 days after injection—B. abortus 80=1:2000; B. melitensis 11=1:1000.

April 28, or 25 days after injection—B. abortus 80=1:4000+; B. melitensis 11=1:1000.

May 7, or 34 days after injection—B. abortus 80=1:600 (inhibition zone 1:200); B. melitensis 11=1:400.

May 24, 1919, or 51 days after injection—B. abortus 80=1:1000 + + +; B. melitensis 11=1:600 + + +.

Skin tests conducted May 7 and 22 with aborto-and melitensis protein were negative. May 24, the animal was chloroformed and cultures carefully made, 51 days after the intravenous infection.

Postmortem Examination.—The cadavar was slightly emaciated and weighed 1,680 gm., a loss of 200 gm. The spleen was normal in size and light in color, and the lymph nodes were distinctly enlarged and rather firm. The liver, kidneys, and lungs appeared normal. A small superficial ulceration was found on the mucous membrane of the rectum. This lesion was probably caused by the careless insertion of the thermometer. Numerous trichocephalus worms were noted in the appendix and colon. Cultures were made from the heart blood, spleen, liver, kidneys, bile, mesenteric, mediastinal, femoral and axillary lymph nodes, bone marrow, lungs and urine. The agar slants inoculated with liver, spleen, right and left kidneys and femoral lymph nodes were densely crowded with colonies of B. abortus.

Exper. 2.—*Monkey* 11.—Pithecus syrichta (Linn.), male, weight 2180 gm. Agglutination tests repeatedly were negative to B. abortus and B. melitensis. Aug. 6, 1919, at 2:00 p. m., the animal was inoculated intravenously with ½ slant or 16 billion B. abortus No. 14 (isolated from a fetal membrane of a cow in 1917). The monkey never suffered from fever and was always active. The following serum reactions were recorded during the course of the infection:

Aug. 21, or 15 days after injection:

B. abortus 80=1:10—1:100—0.
1:200—1:2000 + + +.

B. melitensis 2=1:10—1:60—0.
1:80—1:800 + + +.

B. melitensis 5=1:40—1:6000 + + +.

B. melitensis 3, 6 and 1=no agglutination.

Sept. 20, 1919, or 45 days after the injection:

B. abortus 80=1:2000 + + +, clumps.

B. abortus 14=1:40—1:80 neg. 1:100—1:1000 + + +.

Nov. 12, or 98 days after the infection:

B. abortus 80=1:80 + + +.

B. melitensis 655, 11 and 614=all negative.

In November, 1919, about 3 months after the intravenous injection of the abortus bacilli, the animal became suddenly ill and was found dead in his cage Nov. 12, 1919.

Postmortem Examination.—Body emaciated, weight 1,750 gm.; spleen small and firm; mesenteric lymph nodes slightly enlarged; tissues rather atrophic and dry; cause of death not determined.

Cultures from heart blood, liver, spleen, kidneys, lymph nodes and bone marrow contained numerous cocci and rods, but no B. abortus.

Exper. 3.—*Monkey* 12.—Pithecus syrichta (Linn.); female, weight, 1,960 gm. Repeated agglutination tests with serum were negative for B. abortus and B. melitensis. Inoculated Aug. 14, 1919, intravenously with ¼ slant or 12 billion B. abortus No. 80 isolated from liver of monkey 2. Temperatures on Aug. 15, 16, 17 and 18, normal. Monkey found dead at 4:00 p. m. Aug. 18, the fourth day after the injection.

Postmortem Examination.—Body well nourished; considerable fluid in abdominal cavity; spleen enlarged and rather soft; liver and kidneys congested; intestines omentum and mesenteric folds injected; lymph nodes in general slightly enlarged and soft. Heart muscle rather flabby, slight ventricular dilatation. Death was probably due to cardiac insufficiency.

Cultures were made from the heart blood, spleen, bone marrow, kidneys, bile, mesenteric, femoral and axillary lymph nodes. B. abortus was recovered from all the cultures except those made with heart blood and bile. The blood plates were densely crowded with colonies.

Exper. 1 must be considered in every respect successful; the monkey reacted to an intravenous injection of a moderately virulent strain of B. abortus by a marked production of specific agglutinins. The temperature curve, which represented the remittent type of pyrexia, resembled in many respects that reported on monkey 47 in Horrock's studies[7] on the conveyance of M. melitensis to healthy animals. The type of temperature was comparable to the one obtained in man, when the patient was suffering from what Shaw designated the "ambulatory" type of Mediterranean fever. The persistence of the infection, which in part explained the high agglutinin titer on the 51st day was proved by the extensive growth of B. abortus on the cultures prepared from the various organs. The observations on monkey 11, from a serologic standpoint, were similar to those noted in monkey 1. The infection exhibited itself primarily by an active production of agglutinins, although the temperature remained normal and the tissues on the 98th day after the injection were found to be free from B. abortus. The negative cultural finding of the tissues after death might in part be the result of the partial overgrowth of the plates and tubes by a coccus, which was probably responsible for the intercurrent disease. In view of the progressively diminishing serum agglutination reached before death, it appeared, however, possible that the monkey had recovered from the infection. Exper. 3 should be repeated in order to prove or disprove our conception that monkey 12 succumbed to a nonspecific injury to the heart muscle provoked by an intravenous inoculation of bacterial protein.

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[7] Report of the Commission on Mediterranean Fever, London, 1905, Part I, p. 50.

The experiments definitely indicate that monkeys are susceptible to B. abortus after intravenous inoculations and that these animals may show febrile reactions similar to those observed in monkeys infected with B. melitensis. The injection calls forth a noteworthy production of serum agglutinins and the bacteria persist for at least 50 days in the liver, spleen, kidneys and lymph nodes, while the blood stream sterilizes itself rapidly.

Infections Produced by the Feeding of Carrots, Bread, or Apples Contaminated with B. abortus of Low Virulence.—In order to prove some of the questions raised in the introduction, it was necessary to make certain experiments by introducing B. abortus into the body via the alimentary tract. Three problems suggested themselves in this connection, namely: 1. Was the single ingestion of a limited number of bacteria sufficient to cause an infection? 2. If not, what was the approximate number of infective feedings and quantity of bacteria necessary to produce a disease similar to malta fever? 3. What were the evidences of a successful feeding infection? It should be borne in mind in studying this problem with B. abortus, that, according to Eyre, McNaught, Kennedy and Zammit,[7] a B. melitensis infection, produced in the monkey by feeding, could not always be ascertained by the course of the animal's temperature or by its general appearance and behavior. In many instances, the postmortem findings and the demonstration of innumerable malta fever organisms in the tissues rudely contradicted the negative clinical or even the serologic observations.

Six monkeys were fed with B. abortus cultures; 5 received the growth of a strain of low virulence, while 1 ingested a highly pathogenic culture of porcine origin. The method of administration of the infective material was quite simple; the bacterial masses of well-grown cultures were smeared on sliced carrots, bread or apples and immediately offered to the animals, by whom the contaminated food was eagerly eaten. The following protocols detail the various experiments:

Exper. 4.—*Monkey* 1.—Pithecus syrichta (Linn.) male; weight, 2,625 gm. March 18 to April 2, 1919, well and active, serum repeatedly examined for B. abortus and melitensis agglutinins with negative results. April 3, 3:30 p. m., fed the growth of 2 peptic digest agar slants of a 48-hour old culture of B. abortus 752 (about 25 billion organisms) smeared on a sliced carrot. The monkey never suffered from fever and the serum reactions conducted on April 20, 28; May 7, 22; June 10 and 25, and July 8, failed to reveal specific agglutinins. On July 20 the animal developed severe diarrhea and was chloroformed in extremis on July 23, or 110 days after feeding the culture.

Postmortem Examination.—Body emaciated, weight 2,025 gm., catarrhal enteritis, enlarged mesenteric lymph nodes, slight spleen tumor and congestion of liver. Cultures were negative for B. abortus, but there was an extensive growth of various intestinal organisms from liver, spleen, lymph nodes and bone marrow. The heart blood was sterile.

The blood serum collected at necropsy agglutinated B. abortus 320 in a dilution of 1:10.

This experiment indicated that a single feeding of a fairly large dose of virus failed to produce an infection in the monkey. It was considered unnecessary to repeat this experiment as similar results were obtained in animals fed with artificially infected milk (exper. 11 and 12).

Exper. 5.— *Monkey* 3.—Pithecus syrichta, male, weight, 2,280 gm. Had been used in exper. 13, in which he had received repeated feedings of goat's milk containing large numbers of B. abortus, with negative results; blood serum repeatedly negative for B. abortus and B. melintensis. From Oct. 4 to Nov. 10, 1919, the monkey was fed daily the growth of one slant each of B. abortus 80 and 14, or an approximate total of 2,270 billion bacteria smeared on sliced carrots or bread. The serum tested Oct. 25, the twentieth day of feeding, was negative. The temperature was normal until Nov. 10, when it rose to 41.5 C. and remained at this level 24 hours. On the morning of Nov. 12, the 36th day of the experiment, the monkey was found dead.

Postmortem Examination.—The body was well nourished; mucous membranes were slightly anemic. Lungs showed numerous bronchopneumonic areas. The spleen was normal in size and consistency. Liver, kidneys, mesenteric, femoral and subscapular lymph nodes were normal.

Cultures of the lungs gave a pure growth of B. bronchisepticus; the lymph nodes and liver showed streptococci; while the blood plates smeared with the spleen pulp were covered with numerous typical B. abortus colonies together with a few streptococcus colonies. The heart blood was sterile, but the serum agglutinated B. abortus 80 in a dilution of 1:600 + + + and B. abortus 14 in a dilution of 1:800.

Exper. 6.—*Monkey* 13.—Ceropithecus philippinensis, female, weight, 2,225 gm. Aug. 5, 1919, well and active; serum negative for B. abortus and B. melitensis. Fed on August 5, 6, 7, 8, 9, 10, 11, 13, 14, 21, 24 the growth of one slant each of B. abortus 80 and 14 on carrots, bread or apples. An approximate total of 1,300 billion organisms were fed. Marked loss in weight. Temperature was never above 40 C.; the blood serum gave the following reactions:

First day of feeding—B. abortus 80=0.
B. melitensis 2=0.
Weight 2,225 gm.
Sixteenth day of feeding—B. abortus 80=1:40 + + +, 1:80 + +.
B. melitensis 2—0.
Twenty-second day of feeding—B. abortus 80=1:80 + + +.
B. melintensis 11=1:100 + + +.
Weight 1,965 gm.
B. abortus 14=1:100 + + +.
B. melitensis 655=1:100 + + +.
Weight 1,890 gm.

Aug. 26, had severe diarrhea and refused to eat. Aug. 27, no improvement; chloroformed.

Postmortem examination.—The cadaver was emaciated; 5 c c of fluid were found in the abdominal cavity; the mesenteric and the femoral lymph nodes were enlarged and rather soft. The spleen was enlarged, the pulp soft with enlarged follicles. The kidneys, the liver and lungs appeared normal. Numerous oesophagostomum nodules were present in the colon and cecum.

Cultures made from the liver, kidneys, urine, lung, bone marrow, femoral, iliac and mediastinal lymph nodes were sterile or contained a few cocci colonies. The plates smeared with the mesenteric lymph nodes were densely covered by typical colonies of B. abortus. The agar slants and the blood plates seeded with spleen pulp showed 11-20 colonies of B. abortus.

Exper. 7.—Monkey 33.—Pithecus syrichta (Linn.), male; weight, 2,575 gm. Agglutination tests with B. abortus 80; B. melitensis 7 and 11 were repeatedly negative. Feb. 11, 1920, white blood cells 16,400; hemoglobin, 68% Fed beginning March 9, 1920 (on 64 days, over a period of 115 days) one peptic digest agar slant each of B. abortus 80 (certified milk strain) and 14 (fetal membrane strain, 1907) on sliced carrots. Total organisms fed were estimated at 10,000 to 12,000 billion (75-80 billion per slant).

The temperature remained normal during the entire period of observation; there was a drop in the hemoglobin from 68% to 64%. The blood serum gave the following agglutination reactions:

March 9, or the 1st day of feeding—B. abortus 80 = 0; B. melitensis 11 = 0; 7 = 0.
April 6, or the 28th day of feeding—B. abortus 80 = 0; B. melitensis 11 = 0.
April 20, or the 42nd day of feeding—B. abortus 80 = 1 : 100 + +; B. melitensis 11 = 1 : 60; 7 = 0.
May 3, or 55th day of feeding—B. abortus 80 = 1 : 100; heated serum also 1 : 100.
May 17, or 69th day of feeding—B. abortus 80 = 1 : 80.
June 1, or 84th day of feeding—B. abortus 80 = 1 : 100—200.
June 17, or 100th day of feeding—B. abortus 80=1 :80
June 30, or 113th day of feeding—B. abortus 80 = 1 : 100 + + +; 1 : 400 ±.
July 10, or 123 rd day of feeding—B. abortus 80=1 :1,000 +.
B. abortus, hog=1 :800 + +; B. melitensis 11 = 1 : 200 + + +.
B. melitensis 20 = 1 : 200 + + +; B. melitensis 9 = 0;
B. melitensis 615 = 0; B. melitensis 27 = 0.

July 10, the 123rd day after the first feeding and 8 days after the last feeding. the animal was chloroformed and the tissues cultured.

Postmortem Examination.—The cadaver weighed 2,510 gm.; the spleen was of normal size, rather hard and slightly nodular. The follicles were distinctly visible in the fairly dry pulp. The mesenteric, iliac, femoral, prescapular and mediastinal lymph nodes were not visibly enlarged; the liver, kidneys, and other organs appeared normal. The omentum was adherent to the appendix and portions of the colon; one of these adhesions enclosed an abscess, the size of a pea, containing brownish liquid pus. In the colon and appendix wall, numerous nodules the size of split peas, containing Oesophagostomum brumpti, were present. Cultures were made from the heart blood, spleen, bone marrow, liver, suprarenals, kidneys, lungs, inguinal, prescapular, mediastinal, mesenteric and iliac lymph nodes and from one abscess in the omentum and one oesophagostomum nodule. The blood plates smeared with the pus of these abscesses and the spleen pulp gave an abundant pure growth of B. abortus. All of the agar slants prepared with crushed liver remained sterile, with the exception of one tube, which showed 2 colonies of B. abortus. The other tissues proved to be sterile.

Exper. 8—*Monkey* 34.—Pithecus syrichta; female, weight 2,400 gm. Feb. 9 to April 6, 1920, repeated agglutination tests with unheated blood serum were negative for B. abortus. On April 6, the hemoglobin was 80% (Sahli) and there were 14,700 white blood cells. The animal was fed on 82 days, over a period of 142 days, one slant each of B. abortus 80 and 14, or a total of 14,000 to 16,000 billion bacteria smeared on bread or sliced carrots. Feeding was stopped on July 29, 1920. Weight, blood counts, temperature and agglutination reactions were determined at varying intervals. The temperature never was above 40 C., and the hemoglobin factor showed normal fluctuations. The blood serum always gave negative serum reactions with B. abortus and B. melitensis antigens. The animal is still alive and in excellent health.

In order to determine the possibility of an elimination of B. abortus in the feces of monkeys heavliy fed with abortion bacilli, guinea-pigs were inoculated subcutaneously with emulsions of stools, which had been collected and prepared on the 36th, 45th and 52nd days of the experiment. The animals were sacrificed on the 50th to 60th day after the inoculations. Characteristic lesions were absent and no abortion bacilli were recovered from their spleens.

Monkeys 3, 13 and 33 are examples of successful feeding infections with repeated and enormous doses of B. abortus administered on carrots, bread or apples. The cultures used in these 3 experiments were of bovine origin and of moderate virulence as judged by the lesions they produced in guinea-pigs. Monkey 3 had been fed previously for a period of 19 days with naturally infected milk, but failed to show signs of infection. The positive cultural findings at necropsy and the specific and high agglutination reactions can be safely attributed to the ingestion of the cultures. Unfortunately, the observations on monkey 3 were somewhat invalidated by the death of the animal from bronchopneumonia. During the summer vacation monkey 13 was attended by an inexperienced caretaker, and lack of proper cleanliness probably resulted in the development of the severe diarrhea. It is, however, evident from the presence of specific serum agglutinins on the 16th day after the beginning of the administration of abortus cultures, that the monkey was infected previous to the occurrence of the enteritis. The latter can, therefore, not be considered a contributory factor to the abortus infection.

Monkey 34 illustrates the fact that the ingestion of enormous doses of B. abortus is not always followed by an infection, provided the appearance of specific agglutinins is to be chosen as the only reliable antemortem criterion. The necropsy findings of its mate, monkey 33, therefore deserve further discussion. The large intestines and the cecum of this animal were seriously injured by numerous abscesses due to oesophagostomum and the pathologic processes extended into the mesentery and omentum. The majority of these parasitic nodules harbored enormous numbers of viable abortion bacilli. In reviewing the necropsy reports of the monkeys successfully infected by the ingestion of B. abortus, it was noted that monkey 13 also showed oesophagostomum nodules, while monkey 3 was not infected. *Special attention has been given to the significance of this observation in subsequent experiments, but true infections with the B. abortus have been recorded in animals which showed no coincidental intestinal lesions due to parasites.* It is therefore only remotely possible that the parasitic lesions predispose to an invasion of B. abortus via the alimentary canal in monkeys. In future experiments animals which are free from round worms should be chosen. In our experience with 80 monkeys bought during the last 3 years, such a prerequisite is probably not readily obtainable with the animals at present available on the market. These conditions are mentioned because the British Commission seriously considered the possibility that an absolute continuity of the mucous membrane of the alimentary tract of monkeys may in some cases prevent the development of a feeding infection with B. melitensis.

The presumptive evidence of infection has never manifested itself by the presence of clinical symptoms, and the course of the animals' temperatures has been constantly normal. The duration of the incubation period and the course of the infection could therefore only be judged by the appearance and degree of the specific agglutinins, which developed in the blood serum of the monkeys. In this connection it should be emphasized that the serum of monkey 3 on the 20th day and of monkey 33 on the 28th day of feeding failed to agglutinate B. abortus, while on the 36th and 42nd day, respectively, the serum of these 2 monkeys was distinctly positive. These observations correspond in many respects to those recorded by Horrocks, Kennedy and Shaw[8] on monkeys infected by the ingestion of B. melitensis; approximately 29 days elapsed after the feeding of the cultures before agglutinins were demonstrable in the blood serum. This delayed appearance of the agglutinins lends additional support to the contention that the parasitic processes of the intestines do not favor the tissue invasion of the ingested B. abortus. For example, in monkey 3, which had no nodules of oesophagostomum in the colon, agglutinins were absent on the 20th day, while similarly in the parasitized monkey 33, 29-30 days elapsed before positive serum tests were obtainable. They were absent at least 28 days after the beginning of the experiment.

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[8] See table in Report of Commission, 1907, Part 6, p. 46.

In those monkeys in whom serologic evidence of infection was present following the introduction of B. abortus by the alimentary route, absolute proof of pathogenicity was obtainable in every instance by the isolation of the organism, at least from the spleen, if not from numerous other viscera.

In order to test the assumption that a strain of B. abortus, which is usually fatal in relatively small amounts to guinea-pigs, possesses also greater invasive properties for monkeys than a less virulent strain, two additional experiments were carried out. The B. abortus strain employed had been isolated by Dr. J. Traum from the liver of an aborted hog fetus and had proved very lethal for guinea-pigs; it grew profusely on glycerol peptic digest agar and proved biochemically and serologically a typical B. abortus.[9]

Exper. 9—Monkey 43.—Pithecus syrichta (Linn.); female, weight 2,375 gm.; healthy, with a blood serum negative to B. abortus and B. melitensis, was fed on 43 alternate days from June 1, to July 29, 1920 (over 58 days) the growth of one slant of B. abortus (hog strain, isolated by Traum) cultivated from the liver of monkey 40. The individual dose averaged 60 billion and for the entire feeding period a total of 2,580 billion bacteria were fed on sliced carrots. The temperature remained normal, but there was a decided loss in weight and specific agglutinins developed in the blood serum as follows:

1st day—weight 2,775 gm.—agglutination with B. abortus=0.
16th day—weight 2,450 gm.—agglutination with B. abortus=1:80 + + +.
37th day—weight 2,425 gm.—agglutination with B. abortus=1:400 + + +.
55th day—weight 2,100 gm.—agglutination with B. abortus=1:800 + + + to 1:1,000 + +.
58th day—feeding stopped.
65th day—weight 2,225 gm.—agglutination with B. abortus=1:600 + + + to 1:2,000 +.

The monkey exhibited no clinical symptoms of infection and was chloroformed on Aug. 4, 1920.

Postmortem Examination.—Body well nourished; spleen slightly enlarged and nodular, pulp rather dry; liver normal in size and color; inguinal, mesenteric, subscapular and mediastinal lymph nodes slightly enlarged and slightly pigmented; both kidneys and bone marrow normal. Principle lobe of the left lung contained an echinococcus cyst the size of a plum. In the colon were numerous oesophagostomum nodules with adhesions to the omentum. Cultures were made from the spleen, liver, kidneys, bile, bone marrow, femoral, mesenteric, mediastinal and subscapular lymph nodes, several oesophagostomum abscesses, urine and heart blood. One of 3 plates spread with spleen pulp gave 2 colonies and one plate of 2 smeared with the juice of the mesenteric lymph nodes showed 8 colonies of B. abortus. All the other cultures were sterile or contained a few staphylococci; on the lung plates B. bronchisepticus colonies were present.

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[9] Jour. Infect. Dis., 1920, 27, p. 185.

Exper. 10—Monkey 44.—Pithecus syrichta (Linn.); male, weight 2,800 gm.; healthy with a blood serum negative to B. abortus and B. melitensis; was fed on 43 alternate days from June 1, 1920, to July 29, 1920 (58 days), the growth of one slant of B. abortus (hog strain) isolated from monkey 40. The method of feeding was the same as used for monkey 43. The temperature remained normal; there was a slight loss in weight and the agglutinins developed in the blood serum as follows:

1st day—weight 2,800 gm.—agglutination with B. abortus = 0.
16th day—weight 2,600 gm.—agglutination with B. abortus = 1 : 40 +.
37th day—weight 2,700 gm.—agglutination with B. abortus = 1 : 100 + + +;
55th day—weight 2,750 gm.—agglutination with B. abortus = 1 : 800 — 1 : 1,000 + + +.
58th day—feeding stopped.
65th day—weight 2,925 gm.—agglutination with B. abortus = 1 : 800 + + +.
81st day—weight 3,000 gm.—agglutination with B. abortus = 1 : 800 + + +.
B. melitensis 27 = 1 : 600 +++.
213th day—weight 3,125 gm.—agglutination with B. abortus = 1 : 80 + + +.
325th day—weight 3,100 gm.—agglutination with B. abortus = 1 : 10 + + +.

On May 5, 1921, the animal was still alive and in excellent condition.

Monkeys 43 and 44 present some interesting features. Judging by the appearance of the serum agglutinins, both monkeys became infected sooner than those which had been fed with less virulent abortion bacilli. The degree of infection did not manifest itself in the necropsy cultures of monkey 43; very few bacteria grew on the plates prepared from the spleen and mesenteric lymph nodes. In connection with the previous discussion particular attention must be concentrated on the fact that the pus material of several oesophagostomum nodules did not contain B. abortus; they were either sterile or contained a few staphylococci. Monkey 44 gradually recovered from his infection and regained his original weight. The blood serum agglutinins returned to a low level, but even after a lapse of 325 days, a strongly positive reaction in a dilution of 1:10 was readily obtained. These two experiments indicate that a fairly high percentage of monkeys can be successfully infected via the alimentary tract when large and repeated doses of B. abortus are fed on sliced carrots, bread or apples. It has been demonstrated that these infections are associated with strongly positive agglutination reactions and that B. abortus can regularly be isolated from the spleen and other organs. The serum of the infected monkeys agglutinates B. melitensis cultures in slightly lower dilutions than B. abortus. In order to prove definitely the nature of the infection, it is necessary to conduct absorption tests as stated in article 2 of this series.

Feeding Experiments with Cow's Milk Artificially Infected with B. abortus.—In a further attempt to investigate the factors involved in the infection through the alimentary canal, 4 monkeys were fed on pasteurized milk, which had been artificially contaminated with varying amounts of 2 bovine B. abortus cultures, Nos. 80 and 14. The strains used in these experiments were the same as those used in the successful experiments 6 and 7.

Exper. 11—Monkey 35.—Pithecus syrichta (Linn.); female, weight, 2,500 gm. Feb. 9 to March 9, 1920, repeated agglutination tests with unheated serums were negative for B. abortus and B. melitensis 7 and 11; the hemoglobin was 70% and the white blood cells 17,400. The animal received in a pannikin on 52 days over a period of 80 days, 100 c c of pasteurized cow's milk to which had been added on each occasion, just before feeding, the growth of one slant of B. abortus 80 and 14. A total of about 10,000 billion bacteria had been given in the milk. As a rule, the monkey received this mixture late in the afternoon, after a starvation period of 18 to 20 hours and usually lapped up from ½ to ⅔ of the amount offered. Inside of from 30 to 60 minutes, the pannikin was ordinarily found to be empty; the animal received then its regular meal.

The monkey never suffered from fever, and gained in weight during the experiment. Agglutination tests made at regular intervals were always negative. The animal is still alive and in good health.

Monkey 36.—Pithecus syrichta (Linn.); female, weight, 2,450 gm. This animal was treated for the same period in a similar manner as monkey 35, with negative results. The serum tests with B. abortus antigens were regularly negative.

Exper. 12—Monkeys 37 and 38.—Monkey 37: Pithecus syrichta (Linn.); female, weight, 2,075 gm., had been under observation since Feb. 9, 1919; serum tests failed to give positive reactions with B. abortus and B. melitensis antigens. The monkey was fed by a similar procedure as monkeys 35 and 36 for 44 consecutive days with 100 c c of pasteurized cow's milk, to which had been added the growth of 1/100 slant of B. abortus 80 and 14. A total of about 80 to 100 billion bacteria were consumed by the animal during the experiment. She never suffered from fever and never gave specific serum reactions.

On May 14, 1920, or on the 65th day of the experiment, or 21 days after the last feeding of artificially infected milk, the monkey received 0.3 gm. of cascara and the growth of one slant each of B. abortus 80 and 14 on carrots for 10 consecutive days; then magnesium sulphate in milk for one day and aloin pills (2, then 4, and then 6 pills of ¼ grain daily) for 7 days, together with carrots artificially infected with B. abortus cultures. Neither catharsis nor the appearance of specific agglutinins could be provoked.

From July 8 until Aug. 19 (from the 121st to the 163rd day) the monkey received daily 25 c c of goat's milk collected from goat 2. This milk contained very few abortion bacilli, but possessed a high agglutinin content. During this period of the experiment and for several months subsequently, the monkey never had fever, and the blood serum gave negative agglutinin reactions with B. abortus and B. melitensis antigens.

Monkey 38.—Pithecus syrichta (Linn.); a female, weighing 2,000 gm., had been under observation since Feb. 9, 1919; the serum had repeatedly been negative with B. abortus and B. melitensis antigens. This animal was fed infected milk in the same manner and on the same dates as monkey 37. Instead of cascara or aloin, tincture rhubarb was administered as a cathartic and 35-40 c c of goat's milk were given on 42 days over a period extending from May 13 to July 8, 1920.

The monkey never suffered from fever, gained slightly in weight and the blood serum developed no specific agglutinins for B. abortus.

It is evident from the data presented that monkeys 35, 36, 37 and 38 failed to become infected. Repeated observations gave the impression that the animals drank a considerable portion of the contaminated food. It was therefore surmised that monkeys 35 and 36 ingested practically the same number of bacteria as those which had been fed with contaminated carrots. The results, however, indicate that this was not the case; in all probability a larger portion of the milk was spilled than was actually recorded, and the total number of B. abortus consumed was less than calculated. In no other manner can one explain why organisms ingested in milk should be less pathogenic than those fed on vegetables. As the repeated feeding of milk containing two slants of bacteria was ineffective, it was not anticipated that $\frac{1}{100}$th of a slant would be sufficient to cause an infection, so that the negative results in monkeys 37 and 38 were not surprising. There is a remote possibility that these monkeys were refractory. One recalls in this connection that the British Commission succeeded repeatedly in infecting monkeys with B. melitensis after the ingestion of a single quantity of mixed milk containing not more than 5,000 specific bacteria. Monkeys 35, 36, 37 and 38 received and probably ingested with impunity many thousand times the amount necessary to cause a typical undulant fever infection.

These experiments demonstrate another fact. The pasteurized milk contained specific agglutinins; its whey clumped B. abortus in a dilution of 1:200. The milk of goat 2 (see appendix), which was fed for 42 days to monkeys 37 and 38, was similarly potent. On one occasion, the secretion of the right infected udder agglutinated the B. abortus in a dilution 1:2,000. Irrespective of the ingestion of this milk, rich in antibodies, neither of the monkeys presented any specific agglutinins in the blood serum. The mere ingestion of milk containing antibodies does not lead, at least in the monkey, to an appearance of agglutinins in the blood serum; only an invasion of the tissues, limited and transitory as it may be, apparently can be responsible for the formation of specific serum agglutinins.

Feeding Experiments with Naturally Infected Goat's Milk.—In this series of experiments an attempt was made to reproduce the conditions as they were found to exist by the British Commission in their feeding experiments with milk obtained from goats naturally infected with B. melitensis. It was originally planned to pass various strains of B. abortus successively through the udders of several goats. In this manner it was hoped to transform the bovine into a caprine strain of B. abortus and perhaps to produce an organism pathogenically even more closely related to the B. melitensis than previous observations had demonstrated. On account of lack of available goats, this phase of the experiment has been temporarily abandoned. The few animals infected with B. abortus furnished milk specimens, which were in many respects similar to those found in malta fever goats. The most important observations dealing with the goats are presented in the appendix to this paper. It is emphasized that the milk repeatedly contained as many as 100,000 to 250,000 viable abortion bacilli per c c. In order to obtain these figures, the milk was plated in suitable dilutions in peptic digest liver agar. It is not unlikely that a large number of viable bacteria failed to grow on the poured plates, irrespective of the fact that the B. abortus strains employed grew ordinarily freely in the presence of oxygen. The figures obtained should therefore be considered as averages; it is readily possible that 1-2 million per c c would be nearer the true content of the milk than the 250,000 recorded. The mid milk and the strippings, after having discarded the first 5 c c, were either sterile or gave a pure culture of B. abortus. The milk obtained from goat 1, which was infected on 3 occasions with 2 different strains of B. abortus, and goat 2, injected once with 3 recently isolated bovine strains were therefore remarkably similar to that ordinarily collected from malta fever goats, and feeding experiments on monkeys, therefore, suggested themselves. The experiments thus far completed are briefly as follows:

Exper. 13—Monkey 3.—Pithecus syrichta; male, weighing 2,150 gm., July 2, 1919, was well and active; the serum was negative for B. abortus and B. melitensis. The animal received for a period of 39 days, in a pannikin, 80-200 c c of milk obtained from goat 1 (see appendix). A total of 2,090 c c of milk were placed in the cage, but the monkey spilled the greater part of the fluid and rarely drank more than 20-30 c c at a time. The milk contained from 50-100,000 B. abortus per c c, and the whey of the right udder agglutinated B. abortus in a dilution of 1 : 1,000. The monkey never had any rise in temperature, and the blood serum when tested on July 18 and Aug. 12 failed to agglutinate B. abortus.

Exper. 14—Monkey 19.—Macacus cynomolgus; male, weight 1,650 gm. Sept. 20 to 30, 1919, serum was repeatedly tested for B. abortus and B. melitensis agglutinins with negative results.

From Sept. 30 to Oct. 30, 1919, the monkey received on 26 week days 60-100 c c of goat's milk (goat 1), obtained from the right uninfected half of the udder. At the beginning of the experiment, the milk from the infected left half of the udder averaged 30-60 c c, was rich in leukocytes and the sediment of a centrifugalized specimen gave when cultivated on glycerol agar slants numerous colonies of B. abortus. Beginning Oct. 12, the secretion of the diseased udder was very scanty, about 5-10 c c and toward the end of the experiment (Oct. 14, 1919) the monkey was fed entirely with milk obtained from the right half of the udder, which agglutinated B. abortus in a dilution of 1:500. The temperature of the monkey remained normal throughout the experiment. Serum tests conducted on Oct. 10 and 25 were negative for B. abortus and B. melitensis. Nov. 8, 1919, after showing symptoms of respiratory disease for 3 days, the monkey was found dead.

Postmortem Examination.—Body emaciated, tissues dehydrated; lungs, numerous bronchopneumonic areas; spleen tumor; liver and kidneys mottled and viable. Mesenteric and femoral lymph nodes slightly enlarged and soft.

Cultures made on blood plates with the heart blood, spleen, liver, bone marrow, kidneys, urine and bile failed to reveal colonies of B. abortus. The lungs, bone marrow, spleen and kidneys revealed hemolytic streptococci.

The experiments clearly indicate that B. abortus infection is not readily accomplished in monkeys fed naturally infected goat's milk. Monkeys 3 and 19, which had been fed for 12-39 days, respectively, the milk of goat 1 failed to develop specific serum agglutinins. One, monkey 3, was definitely not refractory because when subsequently fed B. abortus on carrots he became infected and showed agglutinins in the serum and bacteria in the tissues. In the light of previous observations, the negative results on these two monkeys were probably due to the following factors: 1. The two abortus strains used for the infection of the udder of goat 1 were low in virulence. 2. The numbers of viable abortion bacilli in the milk in comparison to those employed in the carrot feeding experiments were very small. In all probability the daily elimination of bacteria in the milk fluctuated considerably as the figures obtained by direct plating clearly demonstrated. The milk fed to monkey 3 probably contained toward the end of the experiment very few abortion bacilli; thus it was noted that guinea-pigs fed with the same samples of milk failed to contract abortion disease; whereas earlier in the experiment, the milk contained a sufficient number of B. abortus to infect guinea-pigs easily by feeding. Moreover, both monkeys rarely consumed more than one-half of the milk offered; monkey 3 receiving at least at the beginning of the experiment milk specimens, which were teeming with abortion bacilli, spilled the greater portion of the fluid. The negative outcome of these two experiments supports the previous conclusions, that monkeys cannot be infected via the alimentary tract with a relatively small number of abortion bacilli of low virulence even when the contaminated food is administered for more than 30 days. B. abortus, therefore, differs fundamentally from B. melitensis.

Two additional monkeys, 37 and 38 (see data of experiment 12), were fed for 42 days with milk collected from goat 2. It is known that the secretion of this animal contained very few abortion bacilli; the negative outcome of these feeding experiments is probably due to the factors already discussed.

Being impressed by the high virulence for guinea-pigs of a strain of B. abortus recently isolated from a hog fetus, it suggested itself to reinfect goat 1—which was again in a period of lactation having apparently recovered from the original infection with B. abortus 80—with this hog strain and to conduct further feeding experiments on monkeys. These tests are briefly as follows:

Exper. 15—Monkey 40.—Pithecus syrichta (Linn.); female, weight 2,725 gm., had been under observation since Jan. 12, 1920. It had been immunized with living typhoid bacilli, and the blood serum agglutinated B. typhosus in a dilution of 1:8,000, but was negative for B. abortus and B. melitensis. The hemoglobin was 73% and the white blood cells 12,800.

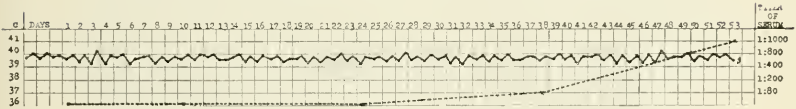

Chart 2 (Monkey 40).—Fed with goat's milk naturally infected with B. abortus of porcine origin.

From March 30 to May 22 the monkey received on 46 successive days 50-100 c c of goat's milk (goat 1), which contained from 20-250,000 abortion bacilli (strain hog) per 1 c c. A total quantity of 2,862 c c of infected milk were offered to the animal in a pannikin. Repeated observations indicated that less than 20 c c were spilled by the animal daily. The method and the time of feeding were those stated for monkey 35. It was estimated that the monkey consumed daily between 1,500 and 2,800 million highly virulent abortion bacilli. On April 23, on the 24th day of feeding, the milk from the right udder of the goat contained 1,500 abortion bacilli per c c and from the left udder 250,000 organisms per c c. May 10, or on the 40th day of the experiment, the milk from the right udder contained 30,000 organisms per c c and from the left udder 180,000 organisms per c c. (For further details see the history of goat 1 in appendix 1.)

The temperature of the monkey showed slight fluctuations during the entire period of observation; it never exceeded the normal average of 40.2 C. (Chart 2). There were no striking clinical symptoms but loss in weight The figures for the hemoglobin percentages, and the agglutination reactions were as follows:

1st day—weight 2,725—hemoglobin 73%—agglutination with B. abortus = 0.
10th day—weight 2,700—hemoglobin 73%—agglutination with B. abortus = 0.
24th day—weight 2,650—hemoglobin 60%—agglutination with B. abortus = 0.
38th day—weight 2,550—hemoglobin 65%—agglutination with B. abortus =
1 : 80 + + +.
50th day—weight 2,350—agglutination with B. abortus=1 :800 + + +.
1 : 800 + + +.
53rd day—weight 2,300—agglutination with B. abortus=1 :800—1 :1,000 + +.
B. melitensis 11 =
1 : 400 + + +

May 1, emulsion of stool was injected into 2 guinea-pigs; when sacrificed 3 months later, these animals were free from abortion disease. On the 53rd day, or 2 days after the last feeding of infected milk, the monkey was chloroformed and cultures carefully made.

Postmortem Examination.—Cadaver in fair condition; the omentum showed old adhesions to the colon. The spleen weighed 7.2 gm., was distinctly enlarged, and firm and exhibited numerous large follicles. Mesenteric, inguinal, subscapular, mediastinal and iliac lymph nodes were enlarged and hard. Liver and kidneys normal; no intestinal parasites or oesophagostomum nodules. In the lung a few small hemorrhages. Cultures were made from the spleen, inguinal, mediastinal, mesenteric, subscapular and iliac lymph nodes, liver, bile, kidneys, urine, bone marrow, lungs and heart blood. The blood plates smeared with the sections or juices of all the organs were densely crowded with B. abortus colonies. The heart blood was enriched in broth and gave on plating a profuse growth of abortion bacilli. The isolated bacteria were promptly agglutinated by a specific abortus serum.

Microscopic examination of the spleen revealed dilated sinuses with a distinct hypertrophy of the sinusoidal cells; the increase of the splenocytes and lymphocytes in the follicles was marked. The malpighian bodies exhibited degenerative changes. In the lymph nodes, desquamated pulp cells and loose endothelial cells in the cortex, together with a pronounced increase of the lymphocytes, attracted attention. Section of the liver, bone marrow and kidneys failed to reveal pathologic changes.

Exper. 16—Monkey 42.—Pithecus syrichta (Linn.); female, weight 2,425 gm. On June 1, 1920, the animal was in excellent health; the blood serum gave no reaction with B. abortus and B. melitensis. For 52 days, over a period of 85 days, the monkey was fed daily 100 c c, or a total of 5,200 c c. of infected milk of goat 1. At the beginning, the animal refused to drink the milk without the addition of sugar; at least one-half of the contents of the pannikin was spilled. In the second week of the experiment, the monkey consumed practically two thirds of the goat's milk placed in the cage.

June 1, or the 1st day of the feeding, the milk from the left udder of the goat contained 11,800 abortion bacilli per c c and from the right udder 2,500 organisms per c c; the milk whey of the left udder agglutinated B. abortus in a dilution of 1 : 200 and from the right udder in a dilution of 1 : 600. On June 25, or on the 25th day of the experiment, the milk of the left udder was sterile, and the milk of the right udder contained 20 abortion bacilli per c c. July 24, on the 53rd day of the experiment, the milk of the right udder was sterile, while the milk of the left udder contained 10 organisms in the sediment of the centrifugalized 25 c c milk sample; the mixed milk of both udders agglutinated B. abortus in a dilution of 1 : 1,000. On August 19, or on the 79th day of the experiment, the milk of the left udder was sterile; the sediment of 25 c c of milk of the right udder gave a few colonies; the whey agglutination was positive in a dilution of 1 : 200.

The serum of the monkey was tested for agglutinins on June 16, July 7 and 28 and August 14, with negative results. There was steady gain in weight and normal temperature throughout the period of observation. The animal died Dec. 20. 1920, while being bled from the saphenous vein.

Postmortem Examination.—Status thymo-lymphaticus; old adhesive left pleurisy; no intestinal parasites. Cultures from spleen, mesenteric, inguinal and subscapular lymph nodes, liver, bone marrow, sterile. Serum gave a partial agglutination to B. abortus in a dilution of 1:60 in unheated serum; negative in heated serum.

The observations made on monkey 40 present several important facts. The ingestion of virulent abortion bacilli supplied in the naturally infected milk of a goat may lead to a generalized invasion of the tissues and even the heart blood. The intestinal tract of monkey 40 was entirely free from parasites or parasitic lesions. The bacterium penetrated the intact mucous membrane. The duration of the incubation period was more than 30 days. The infection manifested itself in the appearance of specific blood serum agglutinins, a loss in weight and a secondary anemia (Basset-Smith [10] reports the constant occurrence of a secondary anemia in undulant fever) with an increase in lymphocytes, while the animal's temperature remained normal, irrespective of the fact that the tissues were teeming with B. abortus. Monkey 42, on the other hand, which also received the naturally infected milk of goat 1, at a later date, showed no signs of an infection throughout the course of the experiment; no traces of agglutinins were demonstrable in its blood serum even up to the date of the termination of the feeding on the 85th day. On the 202nd day, when the monkey died on account of a status thymo-lymphaticus, the unheated serum agglutinated B. abortus in a dilution of 1:60, but specific bacteria could not be isolated from the tissues. The failure to contract an infection was probably due to the fact that the monkey refused to take a sufficient quantity of the infected milk when it was particularly heavily contaminated with abortion bacilli. When the monkey had been trained to drink from 20-40 c c of milk, the total daily number of abortion bacilli consumed was obviously insufficient to cause an infection via the alimentary canal. Exper. 16, therefore, suggests that the ingestion of goat's milk has no predisposing influence in the intestinal invasion of abortion bacilli; it, however, proves by contrast with exper. 15 the previously stated conclusion that only enormous doses of abortion bacilli repeatedly ingested are capable of producing infections in monkeys.

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[10] Jour. Hyg., 1912, 12, p. 497.

Feeding of Pasteurized Cow's Milk Artificially Reinforced with Autolyzed Abortion Bacilli.—The observations of Larson, Sedgwick and Ramsey,[11] of Nicholl and Pratt[12] and of Cooledge[13] on the presence of specific (?) agglutinins for B. abortus in the serum of man, suggested further feeding experiments on monkeys. Cooledge stated that he caused the appearance of B. abortus antibodies in the blood serum of adults by feeding milk, which was naturally infected with B. abortus and which contained B. abortus antibodies. His explanation that these agglutinins and fixing antibodies resulted from an absorption in the large intestines of those present in an infected cow's milk appeared to be not entirely justified. These conclusions drawn by analogy from the experiments of Ehrlich and Wassermann,[14] dealing with the assimilation of antitoxin through the intestinal canal, should not, as will be shown later, be applied without qualification to this problem. In searching for a better interpretation of these serum reactions in man, it suggested itself to investigate the absorption of bacterial proteins through the intestinal tract of monkeys. Cow's, as well as goat's milk, obtained from infected animals, may contain considerable amounts of autolyzed abortion bacilli, which may even exceed the number of viable invasive organisms. In order to prove that in the monkey at least the development of specific agglutinins is not caused by the assimilation of these substances, the following experiments were carried out:

Exper. 17—Monkeys 35 and 36.—These monkeys, used in Exper. 11, with negative results, were fed for 20 days, over a period of 40 days, with pasteurized cow's milk, artificially contaminated with heat killed B. abortus, strain "hog." The cultures were prepared on glycerol peptic digest agar, washed off with distilled water and heated for 2 hours at 60 C. Approximately 65 billion dead bacteria were added to the milk of each monkey. Both animals had been trained to drink the milk and spilled less than 20 c c of the total amount of 50 c c placed in the cage. Serum tests were made every 10 days for 60 days, but no specific agglutinins were recorded.

It is obvious from these experiments that the ingestion of heat killed, partially autolyzed abortion bacilli is not responsible for the appearance of specific agglutinins in the blood serum of monkeys.

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[11] Amer. Jour. Dis. Child., 1915, 10, p. 197.

[12] Ibid., p. 2033.

[13] Jour. Med. Res., 1916, 29, p. 459; Mich. Agric. Exper. Station, 1918, No. 33.

[14] Ztschr. f. Hyg. u. Infektionskrankh., 1892, 12, p. 183.

Such immune bodies are apparently only created as a result of a definite tissue invasion by virulent B. abortus and their presence is, therefore, indicative of a true infection.

EXPERIMENTS WITH B. MELITENSIS

In the three preceding papers of this series, it has been pointed out that the distinction between B. abortus and B. melitensis not infrequently offers great difficulties. Particularly in connection with the B. melitensis infection experiments on guinea-pigs, it has frequently been impossible to state without conducting extensive absorption tests whether a strain of gram-negative bacilli which is agglutinated by a potent B. melitensis or B. abortus serum, is really a B. melitensis.

In order to prove the melitensis character of two of these strains, which were pathogenic for guinea-pigs on intratesticular injection, the following experiments were carried out:

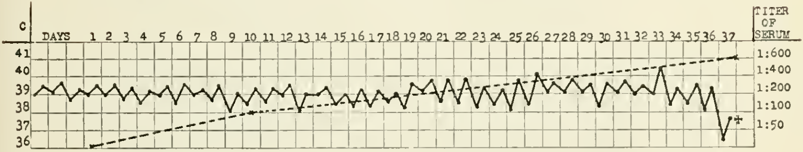

Chart 3 (Monkey 14).—Subcutaneous inoculation of B. melitensis.

Exper. 18—Monkey 14.—Macacus cynomolgus; male, weight 2,830 gm. Aug. 19, 1919, well and active; serum negative for B. abortus and B. melitensis. Aug. 21, inoculated subcutaneously on abdomen with one-half slant of B. melitensis 655 (originally isolated from the precrural lymph node of guinea-pig 655, which had been infected with a culture obtained from the urine and testes of guinea-pig 614, previously injected with B. melitensis, strains 1, 2, 3 and 4). Temperature reaction was distinctly remittant in character (See chart 3). On Sept. 1, or on the 11th day after the inoculation, the blood serum gave the following reactions:

B. melitensis 655 = 1 : 100 + + +
B. melitensis 7 = 0
B. abortus 80 = 1 : 40 + + + ; 1 : 100 +

The monkey appeared lethargic and toxic on Sept. 15; he lost in flesh and was dying Sept. 27; killed with chloroform.

Postmortem Examination.—Body much emaciated (weight 2,250 gm.), spleen small, hard and fibrous; pulp dark brownish; liver slightly mottled; lymph nodes not appreciably enlarged; femoral lymph nodes rather firm; intestinal content frothy; lungs normal.

Cultures were made from the spleen, heart blood, liver, bile, kidneys, bone marrow, mesenteric, femoral, axillary and mediastinal lymph nodes. B. melitensis was recovered from the spleen, right kidney and liver. The blood plates smeared with spleen pulp showed about 100 colonies, those of the right kidney had 20, and those made from the liver gave 2.

The blood serum collected at necropsy, or 37 days after the injection, gave the following reactions:

B. melitensis 655 = 1 : 600 + + +.

B. abortus 60 = 1 : 600 + + +, after absorption with B. abortus 80, serum agglutinated B. melitensis 1 : 400 + + +; the reverse test resulted in negative agglutination with both strains.

B. melitensis 8, 11 and 2—were not agglutinated in dilution 1 : 40 — 1 : 1,000.

Exper. 19—Monkey 15.—Pithecus syrichta (Linn.); male, weight 2,010 gm. Aug. 20 to 28, 1919, well and active, temperature normal; serum repeatedly examined for B. abortus and B. melitensis agglutinins with negative results. Aug. 28, inoculated intravenously with one-quarter slant of B. melitensis 655 (precrural lymph node of guinea-pig 655) by way of right saphenous vein. Temperature was intermittent in character (see chart 4). On Sept. 1, the monkey was noticeably toxic, and had diarrhea. On Sept. 20 a similar reaction was noticed. The blood serum gave the following reactions:

Sept. 10, 1919, or 13th day after infection:

B. abortus 80=1:2,000 + + +.
B. abortus 14 = 1 : 600 + + +
B. melitensis 655 = 1 : 400 + + +; 1 : 2,000 +
B. melitensis 7 = 0

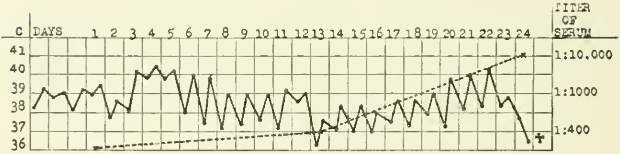

Chart 4 (Monkey 15).—Intravenous inoculation of B. melitensis.

Sept. 20, 1919, or 23rd day after infection:

B. abortus 80 = 1 : 20.000 + + +
B. abortus 11 = 1 : 10.000 + + +
B. melitensis 655 = 1 : 10.000 + + +
B. melitensis 2 = 1 : 1,000
B. melitensis 7 and 9 = 0

On Sept. 21 the animal was comatose and dying. It was chloroformed.

Postmortem Examination.—Body emaciated (weight 1,380 gm.); anus soiled with liquid fecal matter; spleen small, reddish, fibrous pulp; liver light brown and mottled. Mesenteric, femoral and axillary lymph nodes slightly enlarged and soft; bone marrow deep red, like raspberry jam, soft. Blood coagulated rapidly; no intestinal parasites; kidneys light colored. Cultures were made from the spleen, heart blood, liver, kidneys, bone marrow, lung, bile, urine, femoral, axillary and mesenteric lymph nodes. The blood plates smeared with the spleen, bone marrow of left and right femur and one liver lobe were densely crowded with typical colonies; the left lung and urine showed only a few colonies. Absorption tests proved the bacteria to be B. melitensis, group 2.

A study of the microscopic preparations revealed a hyperplasia of the sinusoidal cells similar to that usually seen in an early typhoid infection. The spleen showed slight fibrosis and pigmentation, the latter probably the result of an extensive destruction of the blood cells.

Exper. 20—Monkey 46.—Pithecus syrichta (Linn.); female, weight 2,400 gm. July 27, 1920, injected subcutaneously on left shoulder, 9 billion B. melitensis 27 (strain passed through and isolated from guinea-pigs 880 and 881). The temperature remained normal throughout the period of observation of 94 days. The serum reactions were as follows:

1st day—agglutination with B. abortus 80 = 0
B. melitensis 27 = 0
24th day—agglutination with B. melitensis 27 = 1:200 + + +
B. abortus 80 = 1:400 + + +
94th day—agglutination with B. melitensis 27 = 1:800 + + +
B. abortus 80 = 1:800 + + +

The monkey was chloroformed on Oct. 25, or 94 days after the injection of the culture.

Postmortem Examination.—Body well nourished; spleen small and rather light in color; other organs normal. The omentum, adherent to the cecum, contained two small abscesses; numerous esophagostomum nodules in colon and cecum.

Cultures from kidneys, spleen, bone marrow, urine, liver and lymph nodes sterile; plates smeared with pus from the omental abscess were densely covered with B. melitensis, group 2, colonies.

Monkey 14 showed that a true B. melitensis, when inoculated subcutaneously, provoked a characteristic remittant temperature reaction. Not every monkey, however, reacted in the same manner, and, judging by the clinical symptoms, monkey 46 would have been considered as refractory. Serologic tests and the demonstration of the inoculated bacteria 94 days after the injection proved the animal to have been infected. Monkey 15 responded to the intravenous injection of a comparatively small dose of a strain of B. melitensis by a prompt and marked production of specific agglutinins. The infection, as judged by the animal's temperature, its general appearance and behavior, was very severe and would have ended fatally. Corresponding to the degree of the infection, the tissues were teeming with B. melitensis.

In our paper dealing with the serologic classification of the Brucella group it was shown that one strain of B. melitensis, our No. 20 (obtained from the Lister Institute, Nov. 1919, marked Austria I, received by them from the Royal Army Medical College, Millbank, London), could not be separated by absorption tests from the group I or B. abortus group. In order to prove that strain No. 20 is really a B. melitensis, the following experiment was carried out:

Exper. 21—Monkey 45.—Pithecus syrichta (Linn.); male, weight 2,625 gm. Fed July 26 and 29, 1920, the light growth of a 48-hour old slant of B. melitensis 20 on carrots, a total of about 70 billion organisms. Under observation for 57 days; slight increase in weight; temperature never above normal. Serum reactions developed as follows:

1st day—agglutination with B. melitensis 20 = 0
10th day—agglutination with B. melitensis 20 = 1 : 1,000 + + +
15th day—blood cultures sterile
25th day—agglutination with B. melitensis 20 = 1 : 2,000 + +
B. abortus 80 = 1 : 600 + + +
57th day—agglutination with B. melitensis 20 = 1 : 2,000 + + +
B. abortus 80 = 1 : 4,000 + + +
Chloroformed on Sept. 25, 1920.

Postmortem Examination.—Body in state of excellent nutrition; spleen slightly enlarged and pulp rather soft; other organs normal; no intestinal parasites. Cultures were made from the spleen, liver, kidneys, bile, heart blood, lungs, bone marrow of femurs, mesenteric, inguinal and subscapular lymph nodes. B. melitensis (group 1) was recovered from the spleen and mesenteric lymph nodes.

The prompt appearance of agglutinins in the blood of monkey 45 following the ingestion of two slants of B. melitensis 20 indicates the high invasion properties of this group of bacteria. Moreover, the presumptive evidence is made absolute by the demonstration of the organisms in the spleen and lymph nodes. Cross agglutination and absorption tests proved the isolated strain to be a true melitensis representative of group 1.

It was not considered necessary to repeat experiment 21; the evidence was conclusive that a true melitensis strain differed from a B. abortus strain by its marked invasive properties, when administered by mouth. Two feedings were sufficient to parasitize a monkey, but apparently were unable to produce a characteristic febrile reaction. In the B. abortus experiments, on the contrary, in spite of the fact that a thousand times as many organisms were ingested at each feeding and these repeated over weeks and months, infection only took place when the dosage was tremendous and the bacteria extraordinarily virulent. This can leave no doubt as to the true nature of strain 20, which, while corresponding serologically to the B. abortus group, must be considered a true melitensis organism on account of its marked pathogenicity. This observation is quite in accord with those reported by the British Commission,[15] namely, numerous monkeys in their carefully controlled test series manifested neither febrile reactions nor other clinical manifestations, and yet their blood and organs were literally teeming with B. melitensis. Future studies on the pathogenicity of the representatives of the Brucella group should take these facts into consideration.

One cannot obviously expect the B. abortus to be pyrogenic when entering the tissues via the intestinal tract when the highly pathogenic and invasive B. melitensis quite frequently produces no clinical symptoms. The development of specific agglutinins in the blood serum and the bacterioscopic demonstration of the bacteria in the tissues can be accepted as evidence of true infection.

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[15] Report of Commission, 1907, Part 6, p. 67.

TABLE 1

Results of Feeding Experiments

Number of Experimental Monkey	Duration of "Feeding" in Days	Number of "Feedings"	Dose of B. abortus at Each Feeding	Method of Administration	Evidence of Infection		
					Presumptive		Absolute
					Appearance of Serum Reaction, Days	Reaction of Serum	B. abortus Recovered From
1	110	1	1 agar slant	Smeared on sliced carrots or bread	—	Negative	Negative
3	38	36	2 agar slants	Smeared on sliced carrots or bread	36th	1:600-800	Spleen
13	22	11	2 agar slants	Smeared on sliced carrots or bread	16th 22d	1:40–80 +++ 1:80–100	Spleen, mesenteric lymph nodes
33	115	64	2 agar slants	Smeared on sliced carrots or bread	42d 123d	1:100 +++ 1:800 ++	Spleen, liver and parasitic abscesses
34	142	82	2 agar slants	Smeared on sliced carrots or bread	—	Negative	No necropsy
43	58	43	1 agar slant (hog strain)	Smeared on sliced carrots or bread	16th 65th	1:80 1:600–1:2000	Spleen and mesenteric lymph nodes
44	58	43	1 agar slant (hog strain)	Smeared on sliced carrots or bread	16th 81st 213th	1:40 1:800 1:80	No necropsy
35	80	52	2 agar slants	Added to 100 c c of pasteurized milk	—	Negative	No necropsy
36	80	52	2 agar slants	Added to 100 c c of pasteurized milk	—	Negative	No necropsy
37	65	44	2/100 agar slant	Added to 100 c c of pasteurized milk	—	Negative	No necropsy
38	65	44	2/100 agar slant	Added to 100 c c of pasteurized milk	—	Negative	No necropsy
19	38	26	Small number	Naturally infected milk	—	Negative	No necropsy
40	53	46	Large number (hog strain)	Naturally infected milk	38th 53d	1:80 1:1000 +++	All organs, heart blood, bile and urine
42	85	52	Small number (hog strain)	Naturally infected milk	—	Negative	Negative

DISCUSSION

The experiments dealing with the intravenous inoculation of B. abortus cultures need no special comment; they definitely indicate that B. abortus is similar in its action on the monkey to B. melitensis. Moreover, the findings serve as a concrete illustration of the prolonged existence of certain bacteria in the tissues of apparently healthy animals.

The infections produced in monkeys by the ingestion of B. abortus cultures, however, require further discussion. For the sake of clearness the results of the feeding experiments are shown in table 1. It has been definitely proven that the oral administration of B. abortus cultures is followed by an invasion of the tissues and the formation of specific agglutinins, provided enormous doses are fed repeatedly for a period covering at least 11 days. Even when observing these provisions, it becomes evident that some monkeys may be refractory. The feeding of milk either artificially or naturally infected produces invasion only in rare instances. This is probably due to the difficulty entailed in feeding the organisms in sufficiently large dosage by this method. These facts stand in absolute contrast to the experiences of the British Commission when working with B. melitensis. In the majority of their experiments, a single oral administration of not more than 5000 B. melitensis was sufficient to infect monkeys. It is apparent that this striking difference in pathogenicity cannot be explained on the assumption that the animals in the experiments with B. abortus received a smaller number of organisms in the milk than those to whom B. melitensis was administered. On the contrary, they ordinarily consumed several thousand times as many abortion bacilli as are necessary to produce a distinct disease when B. melitensis is used. The criticism that the elaboration of specific agglutinins, which was used as presumptive evidence of infection, may not occur on account of the continuous feeding and the corresponding absorption of B. abortus protein, was carefully considered. This cannot alter the conclusions drawn from these feeding experiments because the serologic studies were not made until the monkeys had been under observation a sufficiently long time after the oral administration of antigens had been discontinued, and, when killed, the animals were cultured thoroughly to demonstrate any exciting focus of infection. These feeding experiments, therefore, offer definite evidence that B. abortus is not only serologically different from the predominant groups of B. melitensis, but also distinctly less pathogenic.

It appears profitable for comparison to review briefly in this connection the feeding experiments which have been carried out by several workers on guinea-pigs, rabbits and calves. Smith and Fabyean were the first to observe that suspensions of B. abortus introduced by mouth produce lesions in guinea-pigs. Schroeder and Cotton[16] noted the development of abortion disease in the same species, when feeding them milk of apparently healthy cows that had become chronic carriers of B. abortus. Similar observations have been made in our laboratory. The pathologic lesions produced in guinea-pigs fed small quantities of B. abortus are slight, but positive cultures can practically always be procured from the spleen and lymph nodes of these animals. A positive agglutination reaction in guinea-pigs that have ingested abortion bacilli has always been indicative of an infection, irrespective of the slight tissue changes noted at necropsy. The same results have not been obtained with the rabbit. Experimental production of an infectious disease via the alimentary canal is peculiarly difficult in this type of animal and only prolonged feeding of large numbers of bacteria may lead to an infection (Besredka, Litch and Meyer). Feeding experiments conducted on guinea-pigs and rabbits are of little significance and practical value when used as presumptive evidence of the pathogenicity of an organism for man.

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[16] Amer. Jour. Dis. Child., 1913, 6, p. 340.

Some of the generally recognized facts on the susceptibility of calves to B. abortus feeding infections deserve consideration. Schroeder[17] and Simms and Miller[18] have determined that calves, at least those which are exposed to infection during the first 3 to 5 months of their lives and are known to have ingested an abundance of infected milk prior to weaning, are immune. The two latter observers base their conclusions on the fact that they are not able to demonstrate the development of specific agglutinins in the serum of these calves subsequent to the feeding of the infected milk. According to Huddelson,[19] serum antibodies specific against B. abortus develop occasionally in calves as a result of ingesting naturally infected milk, provided their intestinal tract is made permeable by an existing enteritis. He states, furthermore, that the administration of noninfected milk contaminated with a culture of B. abortus for a period of twelve weeks results in the temporary development of complement-fixing antibodies. The demonstration of fixing substances without the coincidental presence of specific agglutinins appears unusual. From a serologic standpoint these findings are remarkable in view of the fact, that old and recent experimental and clinical observations have shown, that the transmission of active or passive immunity through maternal milk can only take place between homologous individuals of certain groups of mammals in the first few days after birth. This occurrence is, however, by no means frequent and certainly does not follow any definite biologic law. This peculiar behavior of agglutinins is in striking contrast to the positive nursing experiments of Ehrlich and of Römer with antitoxins. The intestinal tract of young animals can probably absorb agglutinins derived from and present in the milk of the same species more readily than those obtained from heterologous ones. Moreover, the review of Römer[20] on this subject emphasizes the fact that instances of definitely proven transmission of human typhoid agglutinins through the milk of nursing mothers to their infants are not available. These observations are inconsistent with Cooledge's conclusions relative to the absorption of heterologous agglutinins through the intestinal tract of man. If these negative feeding experiments of Schroeder, Simms and Miller on calves are confirmed by other observers, it must be concluded that the intestinal mucosa of these animals is not permeable to homologous maternal milk agglutinins nor for the specific abortion bacilli.

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[17] Report U. S. Live Stock Assn., 1919, p. 125.

[18] Jour. Amer. Vet. Med. Assn., 1921, 58, p. 533.

[19] Michigan Agric. Exper. Station Bull., No. 32, 1916.

From an epidemiologic standpoint the failure of calves to develop specific agglutinins following the feeding of infected milk is of tremendous interest, if one appreciates that adult bovine animals must be considered exceptionally susceptible to B. abortus and that infections are believed to result from the ingestion of this organism. It is obvious that important factors predisposing to a B. abortus feeding infection are as yet unknown, but it appears probable that the actual number of ingested bacteria plays an important rôle. The successful feeding experiments of Bang, of MacFadyean and Stockmann and of Zwick and Zeller on adult pregnant cows and goats have been carried out by feeding emulsified placentas or very heavy cultures. In several tests of Zwick and Zeller[21] the oral administration of cultures led rapidly to the development of serum agglutinins; in only one instance, however, to abortion and the demonstration of B. abortus in the tissues of the experimental goats and sheep. Similar observations have been made with B. melitensis by the British Commission. The feeding of goats with infected milk or cultures was invariably followed by the production of specific agglutinins, but the infection could only be confirmed at necropsy in 40% of the experiments. These observations support the contention that the development of serum agglutinins in animals following the oral administration of B. abortus or B. melitensis either in culture or in milk is indicative of an infection and that B. melitensis is more invasive to ruminants, therefore effective in smaller dosage.

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[20] Sommerfeld, Handbuch. d. Milchkunde, 1909, p. 486.

[21] Arb. a. d. k. Gsundhtsamte, 1912, 43, p. 44.

Reference to the dosage necessary for a B. abortus infection naturally leads to a discussion of the "virulence" of these bacteria. It must be admitted that our knowledge on this phase of the problem is most incomplete. Guinea-pigs, the usual test animals, exhibit a variable susceptibility, and carefully controlled quantitative experiments have apparently not been carried out. In addition, there is a tremendous degree of variability in the pathogenicity of the different strains. For example, the so-called "hog strain" of B. abortus employed in our experiments was highly pathogenic. Guinea-pigs inoculated with 2 to 5 million organisms succumbed regularly to a generalized B. abortus infection in from 3 to 4 weeks. The necropsy findings revealed only slight emaciation, a spleen tumor and cloudy swelling of the parenchymatous organs. In the test tube the strains grew readily and abundantly. In comparing a large number of strains and their pathogenicity for guinea-pigs, the impression is gained that growth vigor in the test tubes as a rule runs parallel with a high degree of pathogenicity not only for guinea-pigs, but also for other animals. Opportunity will be afforded to discuss this phase of the problem in a paper dealing with the pathogenicity of B. abortus and B. melitensis for laboratory animals. In the feeding experiments on monkeys these differences in virulence were decidedly in evidence. In the light of these findings it is justifiable to anticipate that future bacteriologic work may reveal a strain of B. abortus, which, in relation to its invasive properties, approaches the B. melitensis group more closely than the strains thus far studied.

These possibilities immediately suggest the important question, "Is B. abortus pathogenic for man?" In previous papers the literature relative to this problem has been reviewed and certain data presented in which the most striking evidence as to pathogenicity for man rests on the following facts: (a) Mohler and Traum,[22] by inoculating guinea-pigs, found B. abortus present in one out of 56 tonsils obtained from children; (b) Larson and Sedgwick,[10] Nicholl and Pratt,[11] and Cooledge [13] demonstrated "specific agglutinins and fixing antibodies in the blood of a small percentage of children and adults, who consumed raw milk; (c) B. abortus belongs to the Brucella group and is closely related to B. melitensis, which is highly pathogenic to man.

The experiments of Mohler and Traum have been repeated by Nicholl and Pratt, with negative results, but aside from this the literature is barren of careful bacteriologic studies of those children's diseases in which B. abortus might have been suspected as an etiologic

[22] Twenty-eighth Ann. Report Bur. Animal Ind., Dept. of Agric., 1911, p. 157.

agent. The aforementioned indirect isolation of B. abortus from the tonsils must therefore be considered the only instance in which this organism has been isolated from human tissues. The value of the finding is seriously invalidated by the fact that spontaneous B. abortus infections occasionally occur in laboratory animals accidentally exposed. Until provision can be made to prevent outside spontaneous infection, a repetition for confirmation of the studies of Mohler and Traum is not feasible.

The demonstration of certain anti-bodies in the blood serum of children and adults deserves further experimental investigation. In view of our negative results in a limited series of about 50 serums obtained from children who consumed raw, certified milk, known to contain B. abortus, the positive findings hitherto reported can scarcely be considered constant. The bacteria of the Brucella group are quite readily clumped nonspecifically. This subject has been well covered in several papers on B. melitensis agglutination by Euzière and Roger, Rouslacroix,[23] Nègre and Raynaud[24] and others. In addition, the occurrence of "inhibition zones" is quite common and the serologist must distinguish between coarse and fine clumping of the bacterial emulsion. Only when these limitations have been carefully noted and the nonspecific agglutinins destroyed by heating to 50 C., as recommended by Nègre and Raynaud, can the reaction be considered specific. The frequent errors in the diagnosis of undulant fever reported in several publications demonstrate that only an agglutination in a dilution above 1 : 100 is indicative of a true infection. We have not as yet tested a sufficiently large series of human serums by means of the complement-fixation reaction to draw conclusions. The negative findings of Williams and Kolmer[25] on 50 serums demonstrate that even when one's technic is correct suggestive reactions are exceedingly rare. The observations reported in the literature, which have been previously discussed, in addition to the feeding experiments on cattle, guinea-pigs, rabbits and monkeys, definitely demonstrate that heterologous antibodies in cow's milk are probably never absorbed in the large intestines of adult human beings. The solution of the problem of human pathogenicity can probably not be reached by serologic studies alone but must depend on extensive tissue cultures.

Considering the close relationship between B. abortus and B. melitensis, it seems remarkable that a B. abortus disease resembling undulant fever has never been recognized in countries in which abortion disease in cattle is common. Several explanations can be offered. B. abortus is unquestionably less pathogenic and invasive than B. melitensis. It is a well-known fact that great care must be exercised in handling cultures of the B. melitensis as bacteriologists working with the organism have become infected in an unusually high proportion of instances as compared with other affections. The laboratory infections reported by Carbone,[26] by Birt and Lamb [27] and by Strong and Musgrave [28] are in striking contrast to the absence of instances dealing with B. abortus infections either among laboratory workers, farmers or veterinarians, who unquestionably come in most intimate contact with large numbers of virulent abortion bacilli. Alice C. Evans points out that the number of virulent B. abortus, which persist in the milk of cows is not great, probably even so small as to be negligible from the standpoint of food infection. On the other hand, the milk of malta fever goats is usually teeming with bacteria, in some instances more than 1 million per c c. It is recalled that goats artificially infected with abortion bacilli may discharge enormous numbers of bacteria in their milk, while according to Shaw [29] cows naturally infected with B. melitensis (?) may eliminate comparatively few bacteria. The sediment of 10 to 15 c c milk, which had been centrifugalized, gave for one cow on 7 occasions 3-40 colonies, and from another 7-231 colonies. These observations either indicate that the number of B. melitensis in cow's milk is relatively small or, what is more likely to be true, that the bacteria demonstrated by Shaw were abortion bacteria, which he could not differentiate with his agglutinating serum from B. melitensis. It is probably, however, not so much the amount of virus necessary to cause an infection as the low pathogenicity of the organism for man which explains our freedom from a disease definitely proved to be due to B. abortus.

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[23] Gaz. d. hôp., 1912, 85, p. 289.

[24] Compt. rend. Soc. de biol., 1912, 72, p. 664.

[25] Amer. Jour. Obst., 1917, 75, p. 193.

In the paper on B. abortus in certified milk it was pointed out that the number of abortus bacilli present in this food was small and the excretion probably variable to a large degree. The assumption that abortion bacilli cannot be demonstrated in cow's milk by direct plating has been refuted by the observations of Huddelson and also of Steck, who isolated the bacteria on ordinary dilution plates. Until our knowledge relative to the actual number of B. abortus organisms in market or certified milk is based on similar detailed tests as reported for milk of malta fever goats, it would be unwise to explain our apparent freedom from "abortion disease" by the low number of viable and invasive bacteria. A sudden increase in virulence as shown in one of our goats may lead to a discharge of enormous numbers of abortion bacilli from the udder.

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[26] Arch. per le sc. med. Torino, 1904, 28, p. 273.
[27] Lancet, 1899, 2, p. 701.
[28] Philadelphia Med. Jour., 1900, 6, p. 996.
[29] Report of Commission, 1906, Part 4, p. 23.

It is impossible to predict and to estimtae the consequences of such an event on the human body. An answer to the question—"Is B. abortus pathogenic for man?"—must therefore be kept sub-judice even if the evidence thus far collected and critically analyzed encourages the belief that B. abortus is under ordinary conditions nonpathogenic.

CONCLUSIONS

Following an intravenous inoculation of B. abortus, agglutinins develop fairly rapidly in the blood of monkeys. The animals may show an intermittent type of fever and lose weight. At postmortem examination it is possible to recover the organisms from the spleen, lymph nodes and liver, while even on the fourth day after the injection the blood stream is found sterile.

By feeding the growth of glycerol agar cultures of moderately virulent strains of B. abortus on carrots, bread or apples in large and repeated doses, it is possible to obtain definite infections in a fairly large percentage of monkeys. The duration of incubation as judged by the appearance of serum agglutinins is always prolonged and at least 30 to 40 days may elapse until such substances can be demonstrated. The infection can be positively proven by the isolation of B. abortus from the spleen and occasionally from other tissues.

The repeated oral administration of massive doses of highly virulent B. abortus cultures causes a rapid development of specific agglutinins; by the 16th day the serum may be active in a dilution of 1:40-80. Interruption of the feeding leads to a gradual diminution of the antibodies, probably on account of the disappearance of the bacteria from the tissues.

Milk of cows heavily contaminated with cultures of B. abortus of low virulence when fed for at least 52 days failed to cause infections The mere ingestion of cow's milk rich in agglutinins never led to an appearance of these substances in the serum of monkeys.

Milk obtained from goats, which had been experimentally infected with B. abortus of low virulence and which contained relatively few bacteria, failed to produce an infection in monkeys even when fed for 12 to 42 days.

By feeding repeatedly the milk of a goat that had been infected by the injection into the udder of a very virulent strain of B. abortus of porcine origin, it was possible to infect one monkey. B. abortus was isolated at necropsy from all the viscera and the heart blood.

Serum agglutinins specific for the bacteria of the Brucella group are formed only in the presence of a definite infection. The ingestion of heat-killed, autolyzed abortion bacilli is antigenically ineffective in monkeys.

Certain strains of B. melitensis, which produce characteristic lesions in guinea-pigs resembling those of B. abortus, when injected into monkeys intravenously give rise to characteristic temperature, positive agglutination reactions, and the organisms are recoverable at the post-mortem examination from various tissues.

One atypical melitensis strain introduced via the alimentary canal was not always pyrogenic, but it stimulated agglutinins and the ingested bacteria persisted for at least 57 days in the spleen and mesenteric lymph nodes.

The microscopic changes produced by a B. melitensis or by a severe B. abortus infection in the monkey resemble those of an early typhoid infection.

SUMMARY

Virulent strains of B. abortus in sufficiently large dosage are pathogenic for monkeys.

B. melitensis is far more invasive than B. abortus. One or two feedings of one-thousandth the amount necessary to cause an infection with B. abortus is sufficient in melitensis infection to parasitize a monkey.

APPENDIX

HISTORIES OF GOATS WHICH FURNISHED INFECTED MILK FED TO THE MONKEYS

GOAT 1

June 17-20,1919: About 820 c c of milk were collected daily (3rd month of lactation); Trommsdorff's test, 0.03%; cultures made with the centrifugalized sediment remained sterile; blood serum and whey agglutination tests with B. abortus and B. melitensis were negative.

June 21: Infected by the injection of the growth of a 24-hour old agar slant of B. abortus 80 into the right teat canal. The inoculated half of the udder was not milked until June 28; the daily temperatures of the goat remained normal.

June 28: The right udder was tender, the posterior portion was hard and nodular; 60 c c of milk were collected from the right and 350 c c from the left udder. The milk of the right udder was colostrum like, rich in leukocytes and contained 80-100,000 B. abortus per c c.

June 29—July 7: Daily 100 c c of milk from the right udder; Trommsdorff, 0.05%; the appearance of the milk was normal; slant cultures showed ∞ B. abortus colonies.

July 8: The blood serum of the goat agglutinated B. abortus 80 in a dilution of 1 : 1,000; the udder was still nodular and tender; 1 c c of milk from the right udder contained approximately 20,000 abortion bacilli; the milk of the left udder was sterile.

July 17: Collected 100 c c of milk from the right udder; 1 c c of the milk contained 50 viable B. abortus; Trommsdorff, 0.02%.

July 18—Aug. 28: Collected daily 110-150 c c of milk from the right udder.

Aug. 13, 15, 21 and 28: Milk sample cultures for B. abortus remained sterile. Milk collection from Aug. 12—Sept. 13 inclusive fed to 4 guinea-pigs; failed to provoke abortion disease in these animals.

Sept. 1-26: Collected daily an average amount of 126 c c of milk from the right, and an average of 238 c c from the left udder; the whey agglutinated B. abortus in a dilution of 1 :80—1 :100 + + +.

Sept. 27: Infected the left udder by inoculating the growth of one slant each of B. abortus 80 and 14 through the teat canal.

Sept. 28: The left udder was enlarged, hot and tender; the goat registered a rectal temperature of 40.4 C. and refused to eat.

Sept. 29: The temperature of the goat was normal; the secretion of the left udder, about 5 c c, consisted of curdled milk, very rich in leukocytes; B. abortus was demonstrated by smears and cultures; the right half of the udder secreted a sterile milk.

Sept. 30-October 10: Collected 5-30 c c of yellowish milk from the left and 100-170 c c of milk from the right udder. Agglutination tests of whey were positive in dilutions of 1 :100—200 + + +.

Oct. 12: Secretion from the left udder less than 1 c c.

Oct. 20. The goat was bred; secretion from the right udder 50 c c.

Oct. 21-Nov. 10: Milk secretion from the right udder declined from 60 c c to 15 c c and on Nov. 11 there was no longer any secretion from the udder.

March 13, 1920: The goat gave birth to 3 dead but full grown kids; organs of the fetuses were found to be free from B. abortus; milk secretion from both udders was excellent.

March 14 and 15: The goat had retentio placentarum; the blood serum agglutinated B. abortus 80 in a dilution of 1 :40; the milk whey of the right and left udders was positive, in a dilution of 1 :10; the cultures made from the centrifugalized milk sediment were sterile.

March 25: Infected right udder by inoculating into the teat canal the growth of one slant of B. abortus ("hog's liver"), about 85 billion organisms.

March 26-April 1: The udder was very tender and edematous; the supramammary lymph nodes were enlarged and firm. The goat had a toxic appearance, ate little, failed to ruminate and lay in a stupor in the corner of the pen. The coat was rough and the temperature rose daily, in the evening above 40.8 C. The secretion collected from the right udder (not more than 50 c c) was blood-tinged, partially curdled, rich in leukocytes and teeming with B. abortus.

April 2-8: The goat improved clinically; the secretion varied between 35-70 c c from the right and 200-310 c c from the left udder. The temperature of the goat was normal.

April 12: The blood serum of the goat agglutinated B. abortus 80, 1 :2000; the milk whey of the left udder was positive in a dilution of 1 :800 and the one of the right udder in a dilution of 1 :2000 + + +.

April 13-August 28: The goat appeared well, gained in weight and secreted between 60-125 c c milk from the right and 200-300 c c from the left udder. The right half exhibited on palpation numerous nodular indurations; similar but less marked areas were noted in the left udder. Both supramammary lymph nodes were the size of large eggs, very hard and fibrous, but not tender; the leukocyte count of the physically normal milk varied between 6-15,000,000 per c c of secretion obtained from the right and the left udder. The agglutination reactions with serum and milk whey are shown in table 2.

TABLE 2

AGGLUTINATION REACTION WITH SERUM AND MILK WHEY

Date	Day after Third Infection	Serum Value	Milk Whey Agglutinates	
			Left Udder	Right Udder
March 25, 1920	0	1:40	1:10	1:10
April 12, 1920	18	1:2000	1:800	1:2000
April 23, 1920	29	......	1:400	1:400
May 10, 1920	46	......	1:1000	1:600
May 22, 1920	58	1:1000	......	......
June 23, 1920	90	......	1:100	1:400
July 31, 1920	128	1:2000	1:1000	1:40

Oct. 25: There was no longer any secretion from the udder. The goat was bred.

March 10: The goat gave birth to 3 mature, healthy kids; the milk collected on March 14, 16, 18 and 20 was sterile; the blood serum aggluinated B. abortus in a dilution of 1:20; the milk whey was negative in a dilution of 1:20.

TABLE 3

NUMBER OF B. ABORTUS ISOLATED FROM 1 C C OF THE SECRETION OF EACH UDDER

Date	Day of Third Infection	Number of B. abortus per c c of Milk	
		Left Udder	Right Udder
March 25, 1920	0	Sterile	Sterile
March 27, 1920	2	Sterile	Dilution too low
April 8, 1920	14	50	40,000
April 23, 1920	29	1,500	250,000
May 10, 1920	46	180,000	30,000
June 1, 1920	68	11,800	4.000
June 25, 1920	93	0	20
July 24, 1920	121	0	10 per loop of sediment
Aug. 19, 1920	147	0	3-5 per loop of sediment
Aug. 31, 1920	159	0	0

GOAT 2

May 27, 1920: Milk whey from both udders gave negative agglutination reactions; blood serum unheated agglutinated B. abortus in a dilution 1:40 + + +; heated serum was negative.

June 30: Infected by direct inoculation into the right teat canal of 5 c c representing the growth of one agar slant each of B. abortus 60, 100 and 11 (all recently isolated and obtained from Dr. Ward Giltner, East Lansing, Mich.).

July 1-8: The goat was perfectly well; the udder was slightly tender and edematous, but the right udder was not milked.

July 9-18: Daily 15-25 c c of milk were collected from the right and 50-70 c c from the left udder.

July 19: Milk directly plated was sterile; the sediment of 15 c c of milk of each udder gave the following number of colonies; right about 48, left 10 colonies per loopful.

July 24: A similar test gave the following result: right 4, left 12 colonies per loopful of sediment.

July 28: The blood serum agglutinated B. abortus 80 in a dilution of 1:2000 + + +; the milk whey of the left udder—1:80 + + +; the milk whey of the right udder—1:2000 + + +.

July 29-Aug. 31: The daily collection of milk gradually dropped from 150 c c to 35 c c. Repeatedly tested, the sediment was found to be sterile, and when inoculated into guinea-pigs failed to provoke abortion disease.

Sept. 10: There was no longer any secretion of milk. Blood serum agglutinated B. abortus in a dilution of 1:800 + + +.

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